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Novel effects of Prefoldin Pathway on Intestinal Homeostasis via Dietary Restriction in Drosophila melanogaster

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Novel effects of Prefoldin Pathway on Intestinal Homeostasis via Dietary Restriction in *Drosophila melanogaster*

By

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A culminating thesis submitted to the faculty of Dominican University of California and Buck Institute for Research on Aging in partial fulfillment of the requirements for the degree Master of Science in Biology

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Overview

The field of medicine research is embroiled in a battle against aging. Particular focus is on the extension of lifespan and health-span. Lifespan duration is affected by many factors, one of which is the maintenance of the intestines of the organism. Homeostasis of the intestines is controlled by the regulation of intestine cell apoptosis and intestine cell proliferation. My research explores the role of two proteins in the regulation of these processes; Prefoldin6 (*PFD6*) and Unconventional Prefoldin RPB5 Interactor 1 (*URI*). Prefoldin (PFD) is a Cochaperone protein complex evolutionary conserved between vertebrates and invertebrates. This complex is formed from six subunit proteins, some of which are known to assist in other cellular functions including cytoskeleton assembly, proteostasis, gene regulation, and DNA repair. *PFD6* and *URI* are two of these protein subunits. However, the role these subunits play in maintaining intestinal homeostasis is currently unknown. Nor is it known if the expression of these proteins varies in different cell types. My hypothesis was that loss of these subunits would increase intestinal permeability, resulting in a loss of longevity. Downregulation of these these subunits would result in novel activation of established pathways to increase permeability. The model organism, *Drosophila melanogaster* (fruit fly), has complex anatomical, physiological, and behavioral characteristics, a relatively short lifespan, established intestinal stem cell lineage, and powerful genetic tools that allow the rapid understanding of the function of these subunits in the gut. My results show that gut-specific Down Regulation of the proteins increases gut permeability, negatively changes intestinal stem cell proliferation, and regulates longevity in the model organism *Drosophila melanogaster*. My results further elucidate the nature of Cochaperone proteins' effects on the extension or abrogation of lifespan, and contribute to research on these proteins in complex organisms.

Introduction

Lifespan and its extension are two of the primary focuses of gerontological research. One avenue to preserving lifespan, discovered by researchers within the past 30 years, is by maintaining a healthy gut (Lee 12-19). Research indicates that many diseases, such as Crohn's Disease (CD) and Inflammatory Bowel Disease (IBD), have causative factors that include dysregulation of gut homeostasis, particularly in the epithelium (König ; Teshima, Dieleman, and Meddings 159-165).

The epithelium of the human intestine is comprised of 7 cell types; Enterocytes (break down food to uptake nutrients), Goblet cells (secrete mucus), Enteroendocrine cells (secrete gastrointestinal hormones), Paneth cells (produce antimicrobial peptides), Microfold cells (samples antigens that are from the lumen and delivers them to the lymphoid tissue for antibody development), Cup cells (no known function), Tuft cells (immune response), and the Intestinal Stem Cells (ISC) from which all of the others are differentiated (Van der Flier and Clevers 241-260). The literature explains that there are several key factors which may contribute to a loss of intestinal homeostasis; an increase in the apoptosis (programmed cell death) of the cells which comprise the epithelial tissue (Edelblum 413--424; Edelblum 413--424; Arrieta, Bistritz, and Meddings 1512-1520) dysregulation of the repair mechanisms (primarily inducing proliferation of ISCs to replace damaged cells) (Van der Flier and Clevers 241-260; Buchon et al. 200-211), dysregulation of the junction genes which mediate the cell-cell connections in the tissue such as *Junctional Adhesion Module (JAM)* (Arrieta, Bistritz, and Meddings 1512-1520; Lee 11-18), and disruptions to the intestinal microbiota (Teshima, Dieleman, and Meddings 159-165; Arrieta, Bistritz, and Meddings 1512-1520; Bischoff et al.).

The *Drosophila* intestine is not so dissimilar from that of a human intestine, both on the physiological and cellular scale (Apidianakis and Rahme 21-30). The main differences between *Drosophila* and mammalian intestines include an abrogation of the length and complexity (Figure 1A), a simplification of the epithelial layer (Figure 1B), and the usage of only 4 cell types instead of 6 (Figure 1B); these being ISCs, Enteroblasts, Enteroendocrine cells, and Enterocytes (Apidianakis and Rahme 21-30). However, as can be observed in Figure 1B, one of the more conserved elements of gut homeostasis is the balance and function of proteins in the intestines.

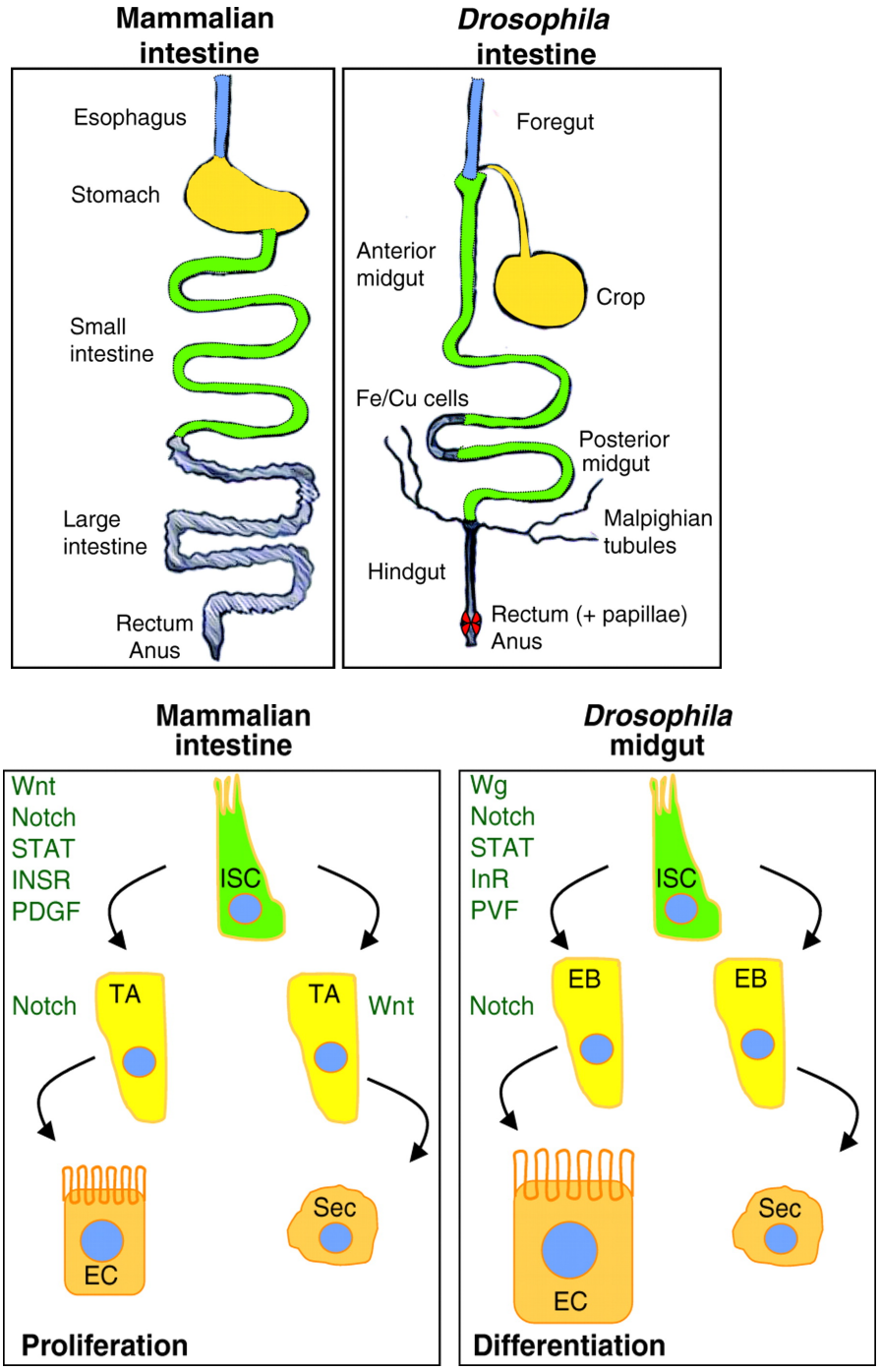

Figure 1: Differences and translatable similarities between mammalian and Drosophila intestines.
Figure 1A (Upper): *Drosophila* and mammalian intestines. Similarly-functioned subsections of the intestine are similarly colored (Apidianakis and Rahme 21-30). Figure 1B (Lower): In mammalian intestines Intestinal Stem Cells (ISCs) differentiate into Transit Amplifying (TA) cells, which differentiate in the presence of notch into the various secretory cells (Sec), or Enterocytes (EC). In *Drosophila*, ISCs differentiate into Enteroblasts (EB), which then differentiate into the secretory Enteroendocrine cells (Sec), or into the Enterocyte cells (EC) (Apidianakis and Rahme 21-30).

Proper protein function is a critical factor for regulating lifespan and promoting longevity (Lee 12-19). Proteins are long chains of amino acids that are formed in cells of every living thing, and an essential part of their creation is how they are folded. Nascent protein chains can often form aggregates unless monitored and correctly folded by molecular chaperone proteins (Hartl and Hayer-Hartl 1852-1858). Though it is inevitable that such misfolded proteins occur in protein construction, formation of aggregates is undesired as aggregated proteins are unsuitable for their biochemical function (Hartl and Hayer-Hartl 1852-1858).

Molecular protein chaperones are one of the ways that cells guide and correct nascent protein chain formation (Dobson and Karplus 92-101; Ellis and Hemmingsen 339-342; Gething and Sambrook 33-45; Hartl 571-579). One such protein that is actively involved in protein folding is Prefoldin. Prefoldin (PFD) (Frydman 603-647), also known as the Gim complex (Hartl and Hayer-Hartl 1852-1858), is actively involved in protein folding in the cytosol of Archaean and Eukaryotic cells (Hartl and Hayer-Hartl 1852-1858). PFD binds to substrates in nascent protein chains (Hartl and Hayer-Hartl 1852-1858), a process which is ATP independent (Hartl and Hayer-Hartl 1852-1858), and assists in stabilizing proteins which have been disrupted and folded differently than their proper 'native' state (forming non-native proteins). In eukaryotes, these non-native proteins are then transported by various macromolecules to an ATP-dependent chaperonin protein, Chaperonin Containing TCP-1 (CCT) (Kubota et al. 89-99). Through this cooperation of protein complexes, PFD and CCT can fold amino acid chains into high-order proteins, most notably including microtubules and actin filaments which form the internal skeleton of cells, or cytoskeleton (Millan-Zambrano and Chavez 10.1098/rsob.140085; Kubota et al. 89-99; Zhao et al. 5857-5865).

Canonical PFD as first discovered in *Saccharomyces cerevisiae*, is a heterohexameric complex that appears similar to a jellyfish (Fig. 2A), with a body comprising of two alpha subunits and four beta subunits that hang off as pairs from either side of the body (Fig. 2B). All subunits feature helical coiled-coil tentacles, the tips of which are partially (~65 Å) unwound to reveal hydrophobic amino acid residues, which are used in the binding of non-native proteins (Hartl and Hayer-Hartl 1852-1858; Siegert et al. 621-632). The alpha and beta subunits differ depending on the if the organism is Eukaryotic or Prokaryotic (Figure 3A and 3C) (Millan-Zambrano and Chavez 10.1098/rsob.140085). Though the PFD complex is relatively well conserved between differing organisms, there are homologs of the PFD complex called non-canonical PFD or PFD-similar complexes that use homologues of the PFD subunits, such as Unconventional Prefoldin RPB5 Interactor (*URI*) (Figure 3C). Because of the essential nature of the PFD complex, however, most of these subunits are replaced by subunits with very similar or highly conserved amino acid sequences (Table 1) (Millan-Zambrano and Chavez 10.1098/rsob.140085; Geissler, Siegers, and Schiebel 952-966). In some Eukaryotic PFD-like complexes, the subunits have been replaced by alternative polypeptides, such as PDRG1 and Monad. However, these homologues still retain similar functions to the canonical (prokaryotic) PFD and are able to cooperate with other Cochaperone complexes (R2TP) (Figure 3C) (Geissler, Siegers, and Schiebel 952-966; Leroux et al. 6730-6743). However, the homologues for subunits of the *Drosophila* genome, are highly conserved relative to those found in the human genome (Millan-Zambrano and Chavez 10.1098/rsob.140085), and thus is suitable for modeling experimentation in this model organism.

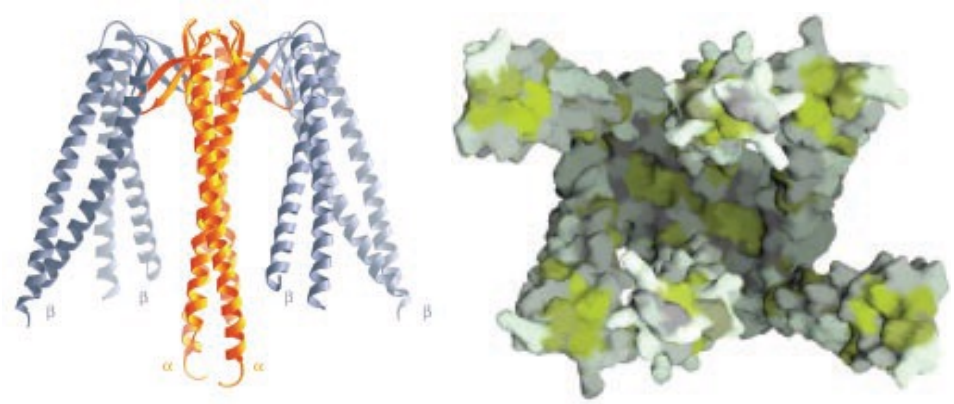

Figure 2: Prefoldin Cochaperon Complex Structure and Binding Domains as Visualized with 3D Simulation Technology

Figure 2A (Left): Canonical Prefoldin heterohexameric complex. Shown are alpha subunits labeled in gold and beta subunits labeled in silver (Siegert et al. 621-632). Figure 2B (Right): Prefoldin shown from beneath, where hydrophobic sections are yellow and hydrophilic sections are grey. (Siegert et al. 621-632)

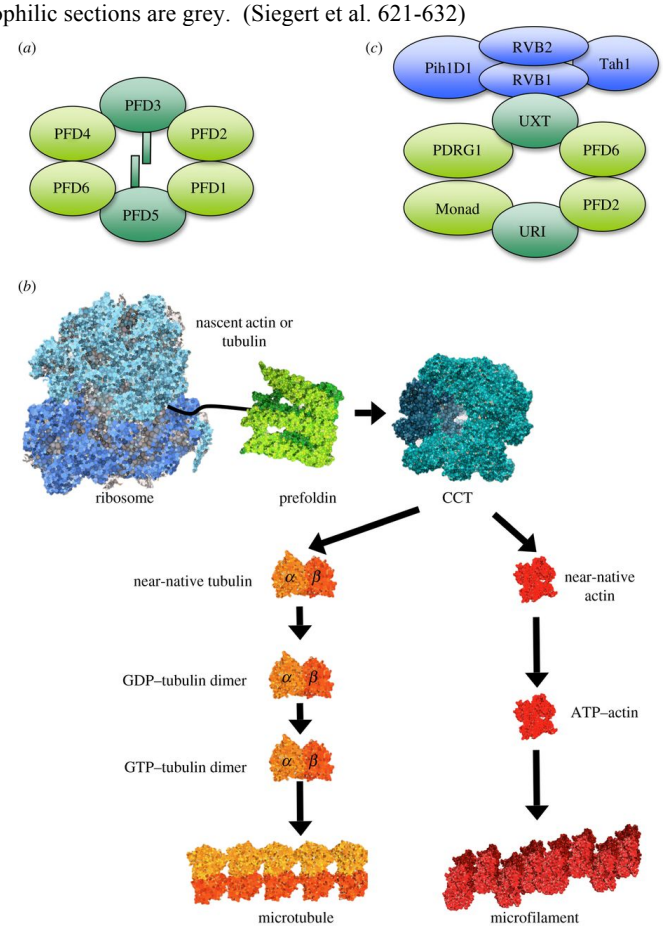

Figure 3: Prefoldin Complex Subunits and Complex Functions

Figure 3A (Upper Left) Canonical Prefoldin as first discovered in *Saccharomyces cerevisiae*, a heterohexameric complex. Two alpha subunits (dark green) are central parts of the structure, along with 4 beta subunits (light green). Figure 3B (Lower) Prefoldin binds unfolded polypeptides and brings them to the ATP-dependent chaperon protein CCT. This co-transport is PFD's primary known function, in addition to its use in folding high-order proteins like microtubules and actin filaments. Figure 3C (Upper Right) In the PFD complex in Eukaryotes, some of the subunits can be replaced in Prefoldin-like complexes. Such complexes are able to interact and cooperate with Cochaperones such as the R2TP complex (purple) (Millan-Zambrano and Chavez 10.1098/rsob.140085; Hartl and Hayer-Hartl 1852-1858).

Subunit found in Mammals (Human)	Subunit found in Drosophila
PFD1	CG13993 (Probable subunit PFD1)
PFD2	PFD2DROME
URI	CG6719 (Probable subunit equivalent *URI*)
PFD4	PFD4DROME
UXT	CG7048 (Probable subunit UXT)
PFD6	*PFD6*DROME

Table 1: Conservation of PFD subunits between mammalian and Drosophila models
Subunit comparison table between *Drosophila melanogaster* and mammals (human). Subunits in *Drosophila* have not all be confirmed, hence are 'probable subunit equivalent' (Millan-Zambrano and Chavez 10.1098/rsob.140085; Guruharsha et al. 690-703).

Most of the described functions of PFD thus far occur in the cytoplasm. However, PFD and PFD-like proteins are found in the nucleus of cells, where it can have different functions (Millan-Zambrano and Chavez 10.1098/rsob.140085). Research has shown the presence of PFD-like structures bearing PFD subunits PFD2 and *PFD6* in the nucleus of prostate cells (Millan-Zambrano and Chavez 10.1098/rsob.140085). Further analysis of *URI* nuclear interactors demonstrated that these subunits can shuttle between the nucleus and cytoplasm, likely together with RNA Polymerase II (Millan-Zambrano and Chavez 10.1098/rsob.140085). In addition to its already known function as a protein chaperone, some subunits of PFD have been found to be active in a variety of other cellular functions (Table 2). For example, the subunit UXT has been observed binding to a transcriptional repressor and suppressing its activity within some cells, differing from the PFD complexes' overall known functions (Kubota et al. 89-99; Zhao et al. 5857-5865; McGilvray, Walker, and Bartholomew 3960-3971). Other PFD subunit functions include cytoskeleton assembly and proteostasis (Millan-Zambrano and Chavez 10.1098/rsob.140085; Mousnier et al. 13615-13620).

Prefoldin Subunit	Interactor (Biological Process)	Other Factors Involved	Organism	Reference
PFD3	HIV integrase	VHL	Human	(Xu and Her 4799-4810)
	hMSH4 (DNA repair)	VHL, p97	Human	(Kim et al. 158-163)
	NF-kB (transcription)	HBx	Human	(Mori et al. 29794-29800)
PFD5	c-Myc (Transcription)	HDAC1-mSin3, TIF1β	Human	(Satou et al. 46562-46567; Kimura et al. 829-836)
		SKp2-ElonginB-ElonginC-Cullin2, Rabring7	Human	(Narita et al. e41891)
	EGR1 (transcription)		Human	(Watanabe et al. 15113-15123)
	P73 (transcription)		Human	(Locascio, Blazquez, and Alabadi 804-809)
PFD3, PFD5	DELLA (gene regulation)		*Arabidopsis thaliana*	(Dorjsuren et al. 7546-7555)
URI	HBx (transcription)	Rpb5	Human	(Delgermaa et al. 8556-8566)
	DMAP1 (transcription)		Human	(Sun et al. 231-244)
UXT	EVI1 (transcription)		Human	(McGilvray, Walker, and Bartholomew 3960-3971)
	NF-kB (transcription)		Human	(Chang et al. 12176-12186)
	HBV EGLF4 kinase (gene regulation)		Human	(Enunlu, Ozansoy, and Basak 471-475)
	ALS2 (gene regulation)		Human	(Markus et al. 670-682)
	androgen receptor (transcription)	VHL	Human	(Chen et al. 55-66; Taneja et al. 13944-13952; Yang et al. 139-153)
		URI	Human	(Mita et al. e63879)
	LRP16 (transcription)		Human	(Rera, Clark, and Walker 21528-21533)
	TAF130 (transcription)		Human	(Chen et al. 55-66)
	Sp1 (transcription)		Human	(Chen et al. 55-66)

Table 2: Subunits of canonical/non-canonical PFD that interact with other nuclear proteins. (Millan-Zambrano and Chavez 10.1098/rsob.140085)

Since many of these functions are associated with proteostasis and actin assembly (Millan-Zambrano and Chavez 10.1098/rsob.140085), a key method of observing their influence or absence is in tissue degradation. In *Drosophila,* lifespan diminution is mediated primarily through tissue degradation of the intestine (Rera, Clark, and Walker 21528-21533). Intestinal barrier dysfunction has shown correlation to a variety of aging-related symptoms in fruit flies, including activation of inflammatory pathways, increased immunity-related gene expression, increased antimicrobial peptide expression, and dysregulation of signaling of the insulin/insulin-like growth factor pathway. These attenuations of function have been observed even in a range of factors such as Dietary Restriction (Rera, Clark, and Walker 21528-21533).

Dietary Restriction (DR) is a robust intervention of nutrition, stating that limiting the uptake of protein, carbohydrates, or amino acids by an organism has the capability of extending its lifespan (Katewa and Kapahi 105-112; Fontana and Partridge 106-118). A diet high in carbohydrates and low in protein has been shown to maximize the elongation of lifespan in mice and in *Drosophila* (Bruce et al. 1129-1135; Solon-Biet et al. 418-430). The conjectured mechanism behind this is the reallocation of resources from reproductive capabilities to somatic maintenance, ens*URI*ng that the organism will survive the shortage of nutrients (Holliday 125-127). The interaction between DR and nutrient pathway Target Of Rampamycin (TOR), a highly conserved serine-threonine protein kinase, which plays a role in regulating cell growth an metabolism, is well established (Zid et al. 149-160). Since its establishment, however, DR has shown unique interactions with several pathways indirectly related to TOR and other proteins (Zid et al. 149-160; Katewa et al. 97-103).

Previous members of the lab used a Lifespan Assay to demonstrate the change in longevity created by loss of the particular gene/protein. This assay is done by placing newly

emerged adult flies (within 48 hours of emerging) on 4 diets. Two on high-nutrient substance (indicated by the red lines), and 2 on low nutrient substance (indicated by blue lines), which will allow for observation of a diet-specific change in phenotype. Of each of the 2 diets, 1 of the foods has a drug (indicated by a '+', or the dotted lines) which will activate a complex found in the flies (more details on this in the methods section), which will cause down-regulation of the targeted gene in a targeted tissue, the other food is lacking this drug (indicated by '-', or solid lines) and thus flies on this food will express the protein at wild-type levels. This previously unpublished data in our lab showed that loss of subunit *PFD6* caused a change in the longevity phenotype upon high nutrient or "Ad Libitum" (AL) diet conditions drastically different than upon DR conditions, making it a candidate gene for this research on pathways of diet-dependent effects on intestinal homeostasis (Figure 4).

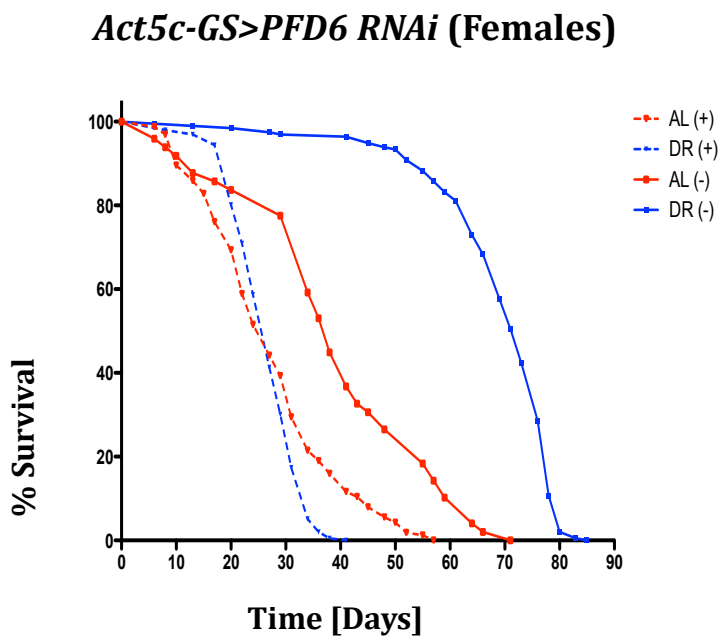

Figure 4: Ubiquitous loss of subunit PFD6 abrogates lifespan.
Whole-body knockdown of *PFD6* using driver Actin5C shows abrogated lifespan in AL+ and DR+ groups, in a significantly diet-dependent manner (Akagi et al.). 200 adult flies were sorted onto 4 diets (AL Drug, DR Drug, AL No Drug, DR No Drug, 800 adult flies in total) 0-1 days after ecclosion. After that, the vials and food were changed every 2 days at which time dead flies were counted. At the end of the assay, the total percentage of dead flies was calculated.

Thus, the aim of this research was to further elucidate the possible functions of subunits *PFD6* and *URI* of the *PFD* complex, which proved to have independent functions from the overall complex. In particular, the majority of this research pertained to phenotypic changes relating to regulation of intestinal homeostasis, and longevity. The hypothesis is that the genes which encode proteins *PFD6* and *URI* play critical roles in maintaining intestinal homeostasis as a means to extend lifespan in *Drosophila*. In addition, it is possible that these genes regulate lifespan by novel mechanisms in established pathways, that is, there are biochemical connections between these proteins which are established between each other, but had not until now been linked to this function and protein complex. In an organized process, it was confirmed in Aim 1

that subunit *PFD6* was essential for longevity by regulation of the intestinal homeostasis in the ECs/EBs of the intestine. In Aim 2, there was begun the process of observing the pathways downstream of subunit PFD6, which may be responsible for the increased intestinal permeability and decreased longevity. Also at the end of Aim 2, possible *PFD* subunit co-modulators of *PFD6* were identified. And in Aim 3, the intestinal and longevity related phenotypes of possible co-modulator subunit *URI* were also identified. By this process, we expanded upon the existing knowledge of the phenotypic effects regulated by the *PFD* complex. By nature of the exploration of this Chaperon complex, this research may unlock potential therapies for protein-related pathologies which may affect intestinal maintenance and the regulation of lifespan.

Hypothesis and Chapters

Hypothesis:

The genes that encode proteins *PFD6* and *URI* play critical roles in maintaining intestinal homeostasis as a means to extend lifespan in *Drosophila*.

Chapter 1:

In aim 1, **the effects of *PFD6* knockdown in different dietary conditions in different cell types of the *Drosophila* intestine were tested.**

Chapter 2:

In aim 2, **downstream targets of subunits of the PFD complex to understand the mechanisms that regulate intestinal homeostasis and lifespan were identified.**

Chapter 3:

In Aim 3, **other subunits of the PFD complex were identified, such as *URI*, other proteins that mediate intestinal permeability were observed, and it was observed if expression levels of these idientifed proteins were altered on different dietary conditions**.

Designs and Methods:

Fly Rearing:

Flieswere reared on a standard laboratory diet (Caltech food recipe; 8.6% cornmeal, 1.6% yeast-extract, 5% sucrose, 0.46% *Drosophila* agar, and 1% acid mix) (Zid et al. 149-160; Kapahi et al. 885-890). Emerged adults were then transferred within 3-5 days of emerging to a yeast extract diet (8.6% Cornmeal, 5% Sucrose, 0.46% Agar, 1% Acid mix, and variable concentrations of yeast extract). The Ad Libitum (AL) diet contained 5% yeast extract while the Dietary Restriction (DR) diet had 0.5% yeast extract. For *Gene-Switch Gal4* drivers, RU486 was dissolved in 95% ethanol and was used at a final concentration of 100μM, where the media was then referred to as '+RU486' (+). The **control** AL or DR diet contained the same volume of 95% ethanol and was referred to as '–RU486' (-). D*URI*ng the study, all flies were raised in 60-60% humidity at 25°C, unless otherwise required for the experiment.

Gene name/type	Source	Stock #	CG #	Genotype
PFD1 RNAi #2	VDRC	102712	CG13993	P{KK112254}VIE-260B
PFD1 RNAi #2	VDRC	18210	CG13993	w[1118]; P{GD7606}v18210
I(2)35Cc RNAi	VDRC	51825	CG15266	w[1118]; P{GD8958}v51825
I(2)35Cc RNAi	VDRC	106186	CG15266	P{KK102158}VIE-260B
mgr(PFD3) RNAi #3	VDRC	27727	CG6719	w[1118]; P{GD12016}v27727
I(3)01239 PFD2 RNAi #3	VDRC	28794	CG6302	w[1118]; P{GD13510}v28794/TM3
PFD4 RNAi #3	VDRC	35481	CG10635	w[1118]; P{GD12596}v35481
PFD4 RNAi	VDRC	101310	CG10635	P{KK107509}VIE-260B
PFD5 RNAi	VDRC	29811	CG7048	w[1118]; P{GD15259}v29811
PFD5 RNAi #2	VDRC	29812	CG7048	w[1118]; P{GD15259}v29812
PFD5 RNAi	VDRC	100796	CG7048	P{KK108708}VIE-260B
PFD6 RNAi	VDRC	34203	CG7770	w[1118]; P{GD10604}v34203
PFD6 RNAi	VDRC	34204	CG7770	w[1118]; P{GD10604}v34204
PFD6.Mut	Bloomington	27476	CG7770	y[1] w[*]; P{w[+mC]=EP}CG7770[G5054]
PFD6 RNAi	VDRC	101541	CG7770	P{KK108999}VIE-260B
UAS-PFD6 OE on 3	Lab made	N/A	N/A	N/A
Rept.TRiP	Bloomington	32415	CG9750	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS00410}attP2/TM3, Sb[1]
Rept.TRiP	Bloomington	36638	CG32464	y1 sc* v1; P{TRiP.GL00598}attP40
Rept RNAi	VDRC	103483	CG9750	P{KK105732}VIE-260B
Spag.TRiP	Bloomington	31253	CG13570	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.JF01194}attP2
Spag RNAi	VDRC	103353	CG13570	P{KK100112}VIE-260B
PiH1d1 RNAi	VDRC	34143	CG5792	w[1118]; P{GD10558}v34143
Pont.TRiP	Bloomington	BL50972	CG4003	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ21078}attP40
Pont RNAi	VDRC	105408	CG4003	P{KK101103}VIE-260B
Uri RNAi	Bloomington	31720	CG11416	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HM04028}attP2
Uri RNAi	VDRC	40674	CG11416	w[1118]; P{GD12627}v40674
Uri RNAi	VDRC	40675	CG11416	w[1118]; P{GD12627}v40675
Daughterless Gene Switch	P. Kapahi	N/A	(Sun et al. 1781-1792)	w1118; P{w[+mW.hs]=GAL4-da.G32},3
5966 Gene Switch	P. Kapahi	N/A	N/A	(Mathur et al. 210-213)
5961 Gene Switch	P. Kapahi	N/A	N/A	(Mathur et al. 210-213)

Table 3: Genotypes and sources of the fly lines used in this research.

Gal4 Gene Switch (GS) system:

In *Drosophila*, GAL4/UAS system (Brand and Perrimon 401-415) is a widely-used method for manipulating transgene expression in a tissue-specific manner. GAL4 is a yeast-derived protein, the expression of which is controlled by tissue/cell type-specific promoters, and it binds to the enhancer region Upstream Activating Sequence (UAS) to allow the transgene expression. In the Gal4-GS system, Gal4:Progesterone fused transgene is expressed under control of a tissue-specific promoter and it can be activated by the progesterone analog RU486, mifepristone (Figure 5). Thus, GAL4 activity is reliant on the presence of the activator RU486, acting as a Gene Switch. Said another way, the UAS is necessary to allow transcription of the transgene it targets, while the GAL4 gene is placed downstream of a native gene promoter, which in this case, is the driver gene line (Da, 5966, 5961). The Gal4 system can only be activated in the presence of the UAS (in its specific tissue/cell), and the entire complex is necessary to drive expression of the transgene. However, this complex as it is would allow for continuous expression of the transgene in the tissue area, meaning there would be no control strain with this same complex. To remedy this, this system has been further modified so that the complex can only be activated by RU486, so that two lines can be present; a control line with the complex, but not expressing the transgene, and the experimental line with the complex and the transgene expressed. In this research, the transgene expressed is primarily an RNA-interference line targeting the specific gene sequence *PFD6* or *URI*.

By utilizing these constructs, one could manipulate the expression of the gene under control of UAS when *Drosophila* were exposed to media with 3.423 mL/L of media RU486 (+), compared to control media lacking RU486 (-). There were 3 different tissue-specific promoters used in these experiments: A promoter that drives ubiquitous expression in the whole body called

Daughterless (Da-GS), an Enterocyte/Enteroblast specific driver (5966- GS), which allowed for observation of phenotypes specific to somatic cells in the intestine (Elliot and Brand 79-95), and a driver specific to Intestinal Stem Cells (5961- GS) to allow for observation of a change in phenotypes in a stem cell lineage in the intestine.

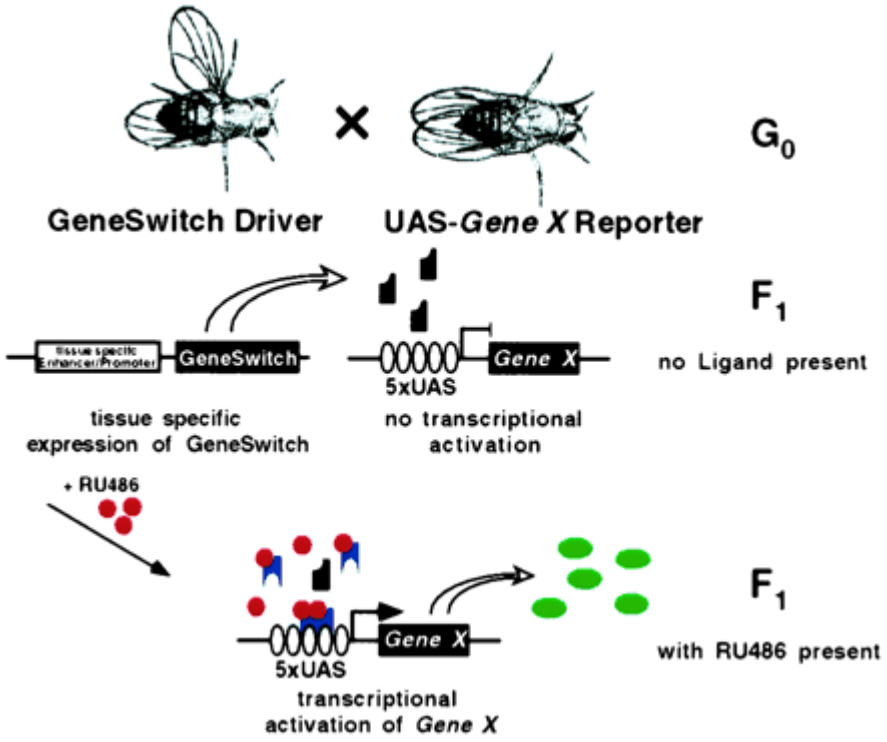

Figure 5: Image example of the UAS-Gal4 Gene-Switch Inducible Promoter System
UAS-Transgene Reporter Flies are crossed with a Gene-Switch Driver Line (5966-GS, 5961-GS, Da-GS). After raising the flies, the adult flies were sorted onto foods which either contained or did not contain transcriptional activating ligand RU486. Those flies with RU486 in the food will had the transcriptional activation of their transgene, leading to its expression in the cell type specific to the Driver line (Osterwalder et al. 12596-12601).

By utilizing these constructs, one could manipulate the expression of the gene under control of UAS when *Drosophila* were exposed to media with 3.423 mL/L of media RU486 (+), compared to control media lacking RU486 (-). There are 3 different tissue-specific promoters

that were used in these experiments; A promoter that drives ubiquitous expression in the whole body called Daughterless (Da-GS), an Enterocyte/Enteroblast specific driver (5966- GS) (Elliot and Brand 79-95), and a driver specific to Intestinal Stem Cells/Enteroblasts (5961- GS).

Smurf Assay:

A Smurf assay is a method for visualizing intestinal permeability in *Drosophila*. Twenty-five female experimental flies were sorted into four to six vials (100-150 flies/experiment) containing paper strips doused with approximately 250 µL of Smurf dye (2.5% FD&C Blue Spectrum dye dissolved in 5% sucrose solution). After 24 hours of feeding with the dye mixture at 25°C, the number of flies that demonstrated a completely blue body, indicating barrier dysfunction, were counted (Rera, Clark, and Walker 21528-21533) (Figure 6). The data was then analyzed for significance using T-Test with Prism 6. This method was selected because it is easy to replicate, and interpretation of results is straightforward, and it is an accurate indicator of disruption in intestinal permeability.

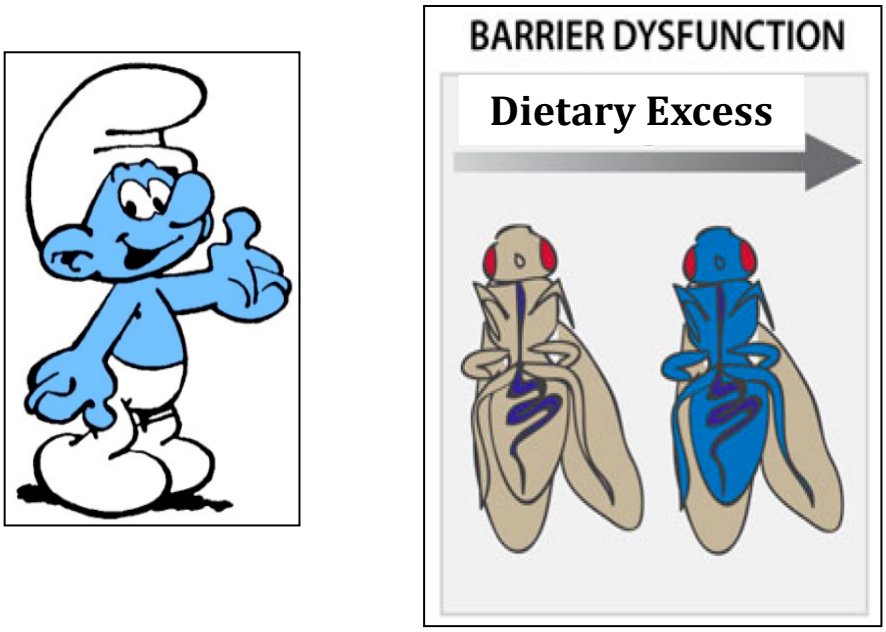

Figure 6: A higher proportion of blue- Dyed flies in the SMURF assay correlates to a higher intestinal permeability (Akagi et al.).
Left: A cartoon depiction of the "Smurf" that gives rise to the name of this assay. Right: With the addition of Dietary Excess of Protein/Carbohydrate, intestinal permeability increases which leads to the escape of the blue dye from the intestine to the rest of the fly's body, turning it blue.

Smurf Assay Over Time:

Similar to the single-time point Smurf assay, this assay allows for an indirect observation of the permeability of the intestines. However, this assay allows the single-time assay to be repeated at multiple time points, to track the change in permeability over time. Twenty-five female experimental flies were sorted into 8 vials (~200 flies/experiment) of differing diet food types. Every 10 days, the flies were swapped onto vials of AL or DR foods containing Smurf dye (2.5% FD&C Blue Spectrum dye dissolved in the appropriate dietary food solutions). After 24 hours of feeding with the dye mixture at 25°C, the number of flies that demonstrated a completely blue body, indicating barrier dysfunction, were counted and the Proportion of Smurf was calculated (Rera, Clark, and Walker 21528-21533). The data was analyzed for significance using T-Test with Prism 6.

Lifespan Assay:

Three to five day-old flies were sorted unto one of 4 possible different medias depending on the assay; AL with RU486, DR with RU486, AL without RU486, and DR without RU486. The fly vials were exchanged every two days, and the number of dead flies was counted as they were exchanged (Katewa et al. 97-103). Data was graphed using Excel 2010/GraphPad Prism 6. The subjectivity of significance is a possible limitation of this method; however, the ease of repeating the experiment, and the ability to use high numbers of flies, gave it a significantly low standard deviation. For this reason, it was a primary and standardized method for visualizing lifespan duration.

Median Lifespan Assay:

After each lifespan assay was concluded, the median lifespan (day) data was analyzed using Prism 6 analysis, and gathered from each assay. After at least 2 of these assays were complete, the median lifespan data was compiled into a separate graph in Prism, and then analyzed with T-Test for statistical significance with Prism 6.

Immunohistochemistry:

Dissected guts were fixed with 4% formaldehyde in 1X PBS for 45 min. Samples were washed three times with PBS for 10 min each, then incubated with 1% NP40/PBS for 30 mins. Samples were washed three times with TBS-TB (0.02 % Triton X-100/PBS, 0.2 % BSA) for 10 min each, and blocking was performed with 5% goat serum in TBS-TB for 2 hours at room temperature. Samples were then incubated with adults primary antibody overnight at 4°C, were washed three times with TBS-TB for 10 mins, and incubated with

secondary antibody two hours at room temperature. Next, the nuclei were stained using DAPI. Samples were mounted with Mowiol mounting media (Sigma-Aldrich) and analyzed by confocal microscope (Zeiss: LSM780).

The following primary antibodies were used in this study: anti-Rabbit phospho-histone H3 (Milipore: 1/500), anti-Rabbit Dcp1 (Cell signaling: 1/500). The following were the secondary antibodies used for signal amplification: Goat anti-rabbit Alexa fluor 488 (Life technologies: 1/500) and Goat anti-rabbit Alexa fluor 555 (Life technologies: 1/500). This method was chosen because it allows for direct visualization of cells that otherwise would have been difficult to visualize; either to phenotype or to quantify.

Apoptotic Cell Detection:

Stock solutions:

Stock solutions was as follows: PBS (usually 1X); Fixative solution: 4% formaldehyde in PBS (freshly prepared); TBS-TX: 20 mM Tris-HCl (pH 7.5), 130 mM NaCl, 1 mM EDTA, 0.1% Triton X-100; and TBS-TB: TBS-TX with 0.2% BSA.

Flies were dissected in PBS solution. Dissected guts were stained with a 5 μg/ml of Acridine Orange/Ethidium Bromide solution (AO/EtBr) for 5 min.. Samples were then washed three times with PBS for 10 min. each, and then fixed with 4% formaldehyde in PBS solution for 45 min. Three more washes with 1X PBS were done, then guts were stained with a 1:4000 DAPI in TBS-TX solution for 15 minutes. Samples were mounted on glass slides with Mowiol mounting medium and visualized using the Red Channel of Fluorescent Microscopy within 25 hours of staining (Liu et al. 3403-3410). Cells were counted via eye, and data was graphed, formatted, and analyzed with T-Test using Prism 6.

Total RNA Extraction:

Tissue samples were dissected from adult female *Drosophila* at differing time points, depending on the experiment. After dissection, samples were homogenized with Lysis buffer (Zymo Research Kit), and lysate was cleared with centrifugation at 10,000 RPM for 1 minute. The supernatant was transferred to a Spin-Away filter and centrifuged into a collection tube with a sample equivalent volume of 95% ethanol. This mixture was then transferred into a Zymo-Spin IIICG column (Zymo Research), where it was centrifuged for 1 minute and the flow through was discarded. An RNA Wash Buffer was added to the column, which was then centrifuged for 1 minute and the flow through discarded. A DNAseI Reaction Mix (6% DNaseI, 10% 10X DNase I Reaction Buffer, 4% DNase/RNase –free H_2O, and 80% RNA Wash Buffer with Ethanol) was added directly to the column matrix, which was incubated at room temperature (~24°C) for 15 minutes and centrifuged for 1 minute. The column was washed once with RNA Prep Buffer, twice with an RNA Wash Buffer, and finally placed into an RNase-free tube, where DNAse/RNase-free water was added, and the tube was centrifuged to collect RNA samples in the tube. With exception of the starting tissue samples, all materials and protocols were from Zymo Research Quick-RNA MiniPrep Kit (Zymogen, Cat#: 11-328). Concentration of eluted RNA was measured by NanoDrop, and the samples were stored at -80°C, or used immediately for Reverse Transcription.

cDNA Synthesis:

1 µg/16 µL of RNA, 4 µL of the iScript RT Master mix, and varying amounts of RNA-se free H_2O were used in each sample, so that the total volume was 20 µL used in each reaction.

The mixtures were spun down and set in a PCR Thermal Cycler for the following cycle conditions: Priming for 5 min at 25°C, reverse transcription for 30 min at 42°C, and reverse transcriptase (RT) inactivation for 5 min at 8°C. Samples were kept on ice for immediate use in PCR amplification or stored at -20°C. All materials, except RNA samples, and protocols were from BioRad iScript Reverse Transcript Kit.

qRT- PCR Preparation:

After defrosting cDNA samples (if necessary), 10 µl of the following mixture was added to each PCR well: Sensi Fast SYBAR Mix (5 µl), forward and reverse primers (0.5 µl each) (sequence varies according to gene), RNase-free water (20%), and prepared cDNA samples (20%). Samples were run through PCR thermocycler for the following times: Priming: 5 min at 25 °C, then Reverse transcription for 30 min at 42 °C, then RT inactivation for 5 min at 85 °C. Data were analyzed and formatted using Microsoft Excel 2010 and GraphPad Prism (T-Test), versions 4 and 6. All materials, except cDNA samples, and protocols were provided by SYBAR Sensi-Fast qRT-PCR Kit (SYBAR).

Primer sequences used:

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
rp 49	CCACCAGTCGGATCGATATG	CACGTTGTGCACCAGGAACT
dMyc	AAATATCCTGCGAGAGGCGG	GAGCGCGATTCGTTCAACTC
upd 3	ACCTACAGAAGCGTTCCAG	GGTTCTGTAGATTCTGCAGG
Diptericin	GGCTTATCCGATGCCCGACG	TCTGTAGGTGTAGGTGCTTCCC
Drosomycin	GAGGAGGGACGCTCCAGT	TTAGCATCCTTCGCACCAG
puckered	CGGGAACGGGGTAAATCCAA	GAGCAGTTACTACCCGCCAG
hid	CGATGTGTTCTTTCCGCACG	TGCTGCCGGAAGAAGTTGTA
Defensin	TTTTGCTCTGCTTGCTTGC	ACATGATCCTCTGGAATTGGA
Rac1	GGAAAGACCTGCCTGCTGAT	TGGGGATAAGACAGTGGCCT

Table 4: Forward and Reverse Primers of the genes tested using RT-PCRs, primers selected by Kazutaka Akagi.

Results:

Chapter 1: *PFD6* is necessary for regulating intestinal permeability and lifespan independent of diet.

Chapter 1.1: *PFD6* is essential for lifespan.

The previously unpublished data (Figure 4) indicated that *PFD6* expression was essential for survival. To confirm this, I used the driver Daughterless Gene Switch (Da-GS) to knockdown *PFD6* expression in the whole body of the fly (Figure 7A), which showed significant abrogation ($p< 0.0001$) of mean lifespan in a diet-dependent manner. Results presented in Figure 7A indicated that systemic *PFD6* knockdown (dotted blue lines AL+) reduced survival, compared to control (solid blue AL-). However, this decrease in survival was more substantial in DR diet (red-colored lines), when comparing the solid red vs. dotted red lines ($P < 0.0005$). Figure 7B invalidated a concern that ubiquitous driver Daughterless (Da) would demonstrate Gal-4 complex "leakage" which might contribute to abrogation of lifespan outside from the effects of gene loss we attempted to demonstrate. Figure 7B shows that flies placed on UAS-Da-GS activating drug food (dotted lines), do not display a significant change in median lifespan, compared to the change in lifespan demonstrated by ubiquitous loss of PFD6 (Figure 7A).

To validate the hypothesis that *PFD6* modulates survival due to its function in the intestine, an intestine-specific driver line was used to knockdown *PFD6* expression in intestinal cells. The 5966-GS (Enterocyte/Enteroblast driver) was chosen because it was important to see the effect of knockdown of *PFD6* subunit in a somatic cell type that makes up at least 90% of the intestinal tissue (Apidianakis and Rahme 21-30). In Figures 7D and 7F, it was observed that knocking down *PFD6* significantly abrogated survival on both diets by at least 20 days in each diet. Thus, this suggested a critical role for *PFD6* in ECs and for lifespan of the flies.

This process was also used to test effect of knocking down *PFD6* in ISCs, using the 5961-GS driver. Interestingly, knocking down of *PFD6* in ISCs did not affect survival a statistically significant manner, regardless of diet (Figure 7D and 7E).

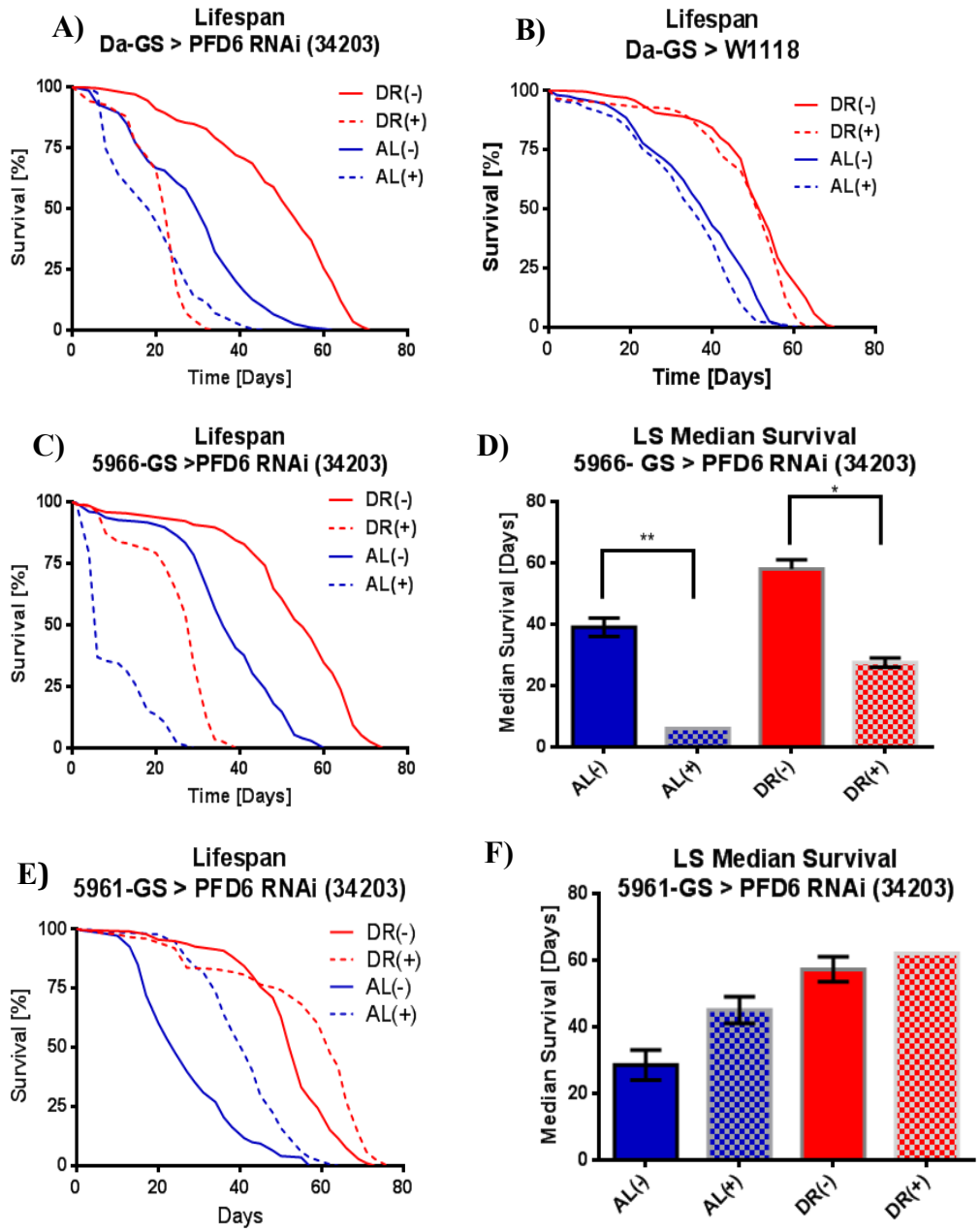

Figure 7: Knockdown of *PFD6* in Da-GS and 5966-GS drivers abrogated lifespan.
Figure 7A: Lifespan (LS) assay of ubiquitous knockdown of *PFD6* subunit using Da-GS driver showed an apparent diet-independent decrease in longevity. Ad Libitum *PFD6* knockdown (AL(+)) demonstrated very strong abrogation of lifespan in comparison to *PFD6* present (AL(-)). Effects of *PFD6* knockdown upon DR appeared to be minimal. Figure 7B: Lifespan assay of ubiquitous Daughterless (Da) driver crossed by control line W1118. This strain is a control strain which demonstrates that Da-GS does not have a significantly negative effect on lifespan by itself. Figure 7C: Enterocyte/Enteroblast-specific knockdown of subunit *PFD6* using 5966-GS driver in longevity assay. There appeared to be a significant difference in the diet-independent abrogations of lifespan. Figure 7D: Lifespan Median Survival graph confirmed that there was a significant decrease in median lifespan upon knockdown of *PFD6* in both diets. Significance of knockdown in AL diets (solid and dotted blue columns) was $P < 0.005$ (**), while significance of decrease in Median Lifespan on DR diets (solid red and dotted red columns) was $P < 0.05$ (*). Figure 7E: Lifespan assay of *PFD6* knockdown in Intestinal Stem cells using 5961-GS driver showed no significant difference in the longevity in either diet. Figure 7F: Lifespan Median Survival graph confirmed that there was no significant difference in lifespan when knocking down *PFD6* in Intestinal Stem Cells. All longevity assays are representative graphs. LS Median Survival graphs are calculated from at least 2 replicates of each lifespan.

Chapter 1.2: Loss of *PFD6* increases intestinal permeability in a diet-independent manner.

My results suggested a critical role for *PFD6* in ECs affected survival (Figure 7), and intestinal homoestasis is known to be a factor in longevity in *Drosophila* (König ; Teshima, Dieleman, and Meddings 159-165). Therefore, I hypothesized that PDF6 knockdown in ECs reduced survival due to its effect on intestinal permeability. To this end, I utilized an established quantitative Smurf assay (Mathur et al. 210-213). In this assay, intestinal permeability can be measured in flies upon feeding them FD&C blue food dye. Flies with permeable intestines are identified due to seepage of the blue dye that results in the entire fly appearing blue (Smurf). Whereas in flies with normal, intact guts, the blue color is isolated to their intestines, rather than their entire body (Un-smurf). The proportion of Smurf vs. Un-smurfed flies in a population can be used to estimate relative intestinal permeability. Flies in each group were maintained on relevant food (AL vs. DR) for 14 days to establish the effect of diet and RNAi knockdown of PDF6. Flies were then transferred to food containing the dye.

My results indicated that systemic knockdown of *PFD6* (with Da-GS driver) significantly increased intestinal permeability, as seen by Smurf assay (Figure 8A). Interestingly, this increase in the intestinal permeability was diet independent ($P < 0.005$ for DR diets and $P < 0.00005$ for AL diets), which supported the lifespan data (see Figure 7). Furthermore, knockdown of *PFD6* in ECs using the 5966-GS driver significantly increased intestinal permeability in the flies (Figure 8B, $P < 0.0005$)). As with the lifespan survival assay, there was no significant change in Smurf levels, regardless of diet, when knockdown of *PFD6* was driven in ICSs alone (5961-GS driver) (Figure 8C). These results, along with lifespan survival assays, strongly suggested a role for *PFD6* in intestinal homeostasis.

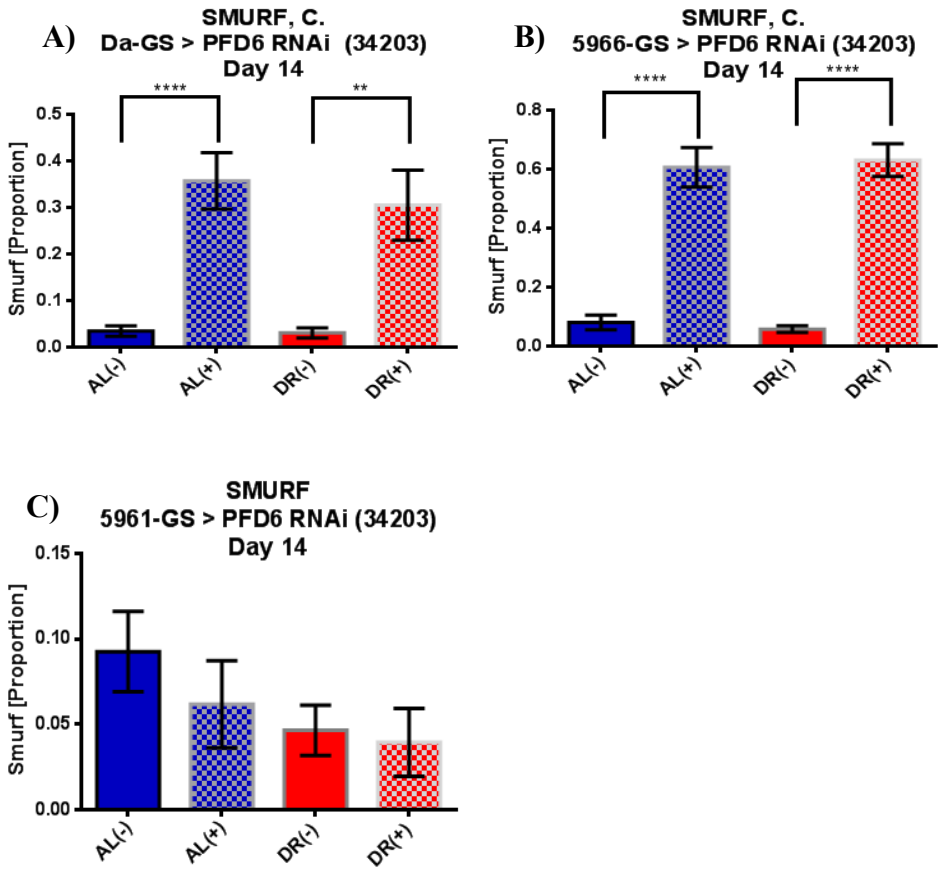

Figure 8: Knockdown of *PFD6* increased intestinal permeability in whole body and in EC/EB.
Figure 8A: Smurf assay of flies with *PFD6* subunit knocked-down ubiquitously using Da-GS driver. Intestinal permeability appeared significantly increased ($P < 0.005$) on DR diet (solid red vs. dotted red columns), though not as significant ($P < 0.0005$) as upon AL diet (solid blue vs. dotted blue columns). Figure 8B: Smurf assay of flies with Subunit *PFD6* knocked-down in Enterocyte/Enteroblast using 5966-GS driver. The difference in the proportion of Smurf upon either diet was extreme ($P < 0.00005$). This correlated well with our previous lifespan survival assays, indicating that the increase in intestinal permeability contributed to the abrogation of lifespan of flies due to *PFD6* knockdown in Enterocytes/Enteroblasts. Figure 8C: Smurf assay of flies with *PFD6* knocked-down in Intestinal Stem Cells. No significant difference was observed, and proportions of Smurf were comparatively low overall when compared to those of *PFD6* knockdown driven by Da-GS or 5966-GS. Graph A and B were compilations of 3 biological replicates, Graph C is a compilation of 2 biological replicates. For consistency of aging-related factors, all Smurf assays were performed at day 14 post sorting onto dietary conditions.

Chapter 1.3: Testing the effects of *PFD6* Knockdown on Intestinal Stem Cell Proliferation.

There is a decrease in lifespan when expression of *PFD6* is knocked-down (Figure 7) via the Da-GS and 5966-GS drivers, and that this is likely caused by the dysfunction of the intestinal epithelium by knocking-down *PFD6* in enteroblasts/enterocytes, which leads to increased intestinal permeability (Figure 8). However, as mentioned before, intestinal homeostasis is regulated by multiple factors, one of which is its rate of repair. This is also referred to as Compensatory Proliferation, as the ISCs proliferate, in a compensatory manner, in response to damage. Increased proliferation can be indication of increased apoptosis, which leads to increased gut permeability, whereby the gut must drive increased proliferation to maintain intestinal homeostasis (Van der Flier and Clevers 241-260). In order to observe the effects on Compensatory Proliferation when knocking down *PFD6*, I used an immunohistochemistry stain for a mitotic marker protein called Phosphohistone-H3 Protein (pH3) (Ayyaz, Li, and Jasper 736-748). pH3 is expressed in stem cells that are actively replicating, thus serving as a marker for proliferation of stem cells (Ayyaz, Li, and Jasper 736-748). For this assay, the flies were placed on their respective diets (AL vs. DR) for 14 days, after which they were dissected. Their intestines were fixed and incubated with the anti-pH3 primary antibody, followed by incubation with a fluorescent-conjugated secondary antibody. Sample collection on Day 14 was chosen to keep results time-standardized with the Smurf assays, thus allowing for direct comparison of data from the two different methods.

When staining flies with *PFD6* knocked-down ubiquitously using the Da-GS driver (Figure 9A), the number of pH3-positive cells was significantly decreased, on both AL and DR diets ($P < 0.0005$, and $P < 0.05$ respectively). This indicated that compensatory proliferation was significantly decreased when *PFD6* was ubiquitously knocked-down. However, when this assay

was performed on flies with enterocyte/enteroblast-specific knockown of *PFD6* (Figure 9B), we saw no significant change in the number of pH3-positive cells, which occured upon both AL and DR diets (Figure 9B). Thus, ISC proliferation was not effected by EC-specific knockdown of *PFD6*.

As stated before, proliferation occurs as a response to damage, and from our intestinal permeability assay, it was possible that cellular damage was occurring, resulting in the increased intestinal permeability (see Figure 8B). Therefore, it could be hypothesized that the loss of *PFD6* was somehow interfering with the signaling pathway that alerted the intestinal stem cells to the fact that damage was occurring. To look further into this matter, I did an anti-pH3 staining on flies with *PFD6* knocked-down in intestinal stem cells (Figure 9C). The results showed a decrease in the number of pH3-positive cells on the AL diet (solid circles vs. blue squares), indicating that flies with this knockdown condition had a significantly decreased ($P < 0.05$) ISC proliferation response. There was no significant difference in the number of pH3-positive cells between the control and knocked down flies on the DR diet.

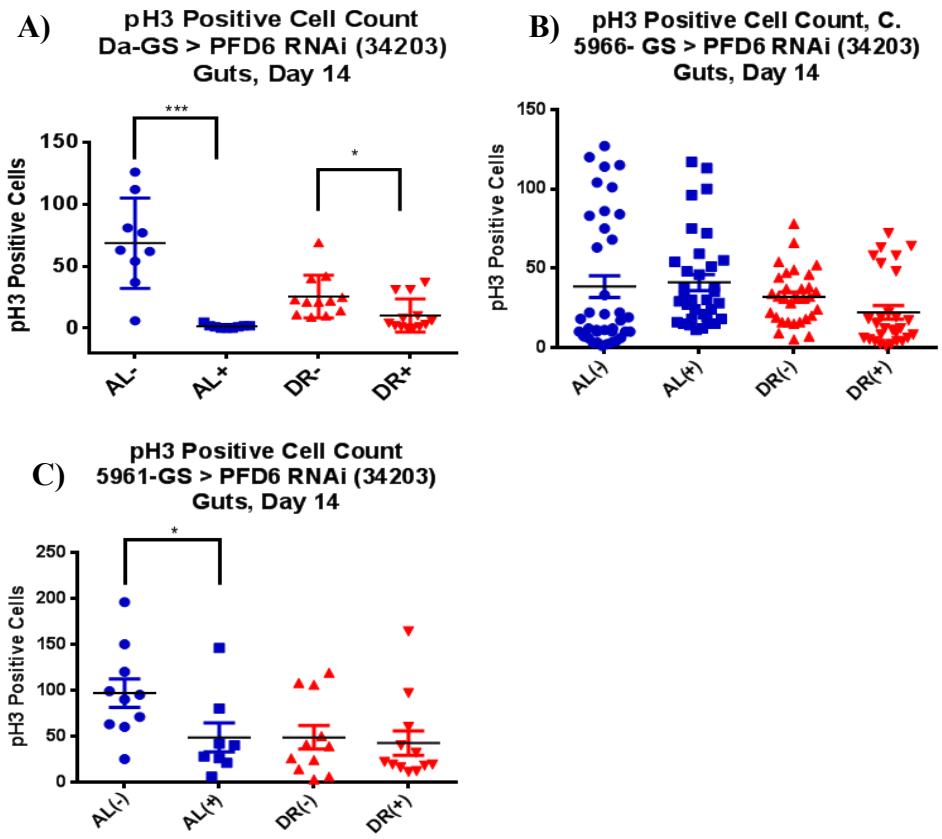

Figure 9: Ubiquitous loss of *PFD6* decreases stem cell proliferation, while loss of *PFD6* in ISC decreases proliferation in a diet-dependent manner.
Figure 9A: Ubiquitous knockdown of *PFD6* using Da-GS decreased proliferation, as indicated by count of pH3 positive (proliferating) stem cells on AL (blue) and DR (red) diets in a significant manner ($P < 0.0005$ and $P < 0.05$, respectively). Figure 9B: Enterocyte/Enteroblast-specific knockdown of *PFD6* using 5966-GS did not affect proliferation in a significant manner. Figure was formed of 3 different biological replicates. Figure 9C: Intestinal Stem Cell-specific knockdown of *PFD6* using 5961-GS decreased proliferation on AL diet (blue circles and blue squares) in a significant manner ($P < 0.05$). For consistency of aging-related factors, all Smurf assays were performed at day 14. All samples are intact intestines.

Chapter 2: Loss of longevity from loss of *PFD6* may be contributed to by increased inflammation and infection from increased intestinal permeability.

Having established that loss of *PFD6* in EC demonstrate the highest significant effect on intestinal homeostasis, and therefore lifespan, the next step was to investigate the pathways responsible for regulating the cellular cycles that lead to intestinal dysregulation or maintenance. The first way to do this was to evaluate already known pathways involved in tissue maintenance: cell apoptosis and cell proliferation. Using the PFD knockdown constructs used in the previous chapter, I utilized qRT-PCR to analyze gene expression of key genes within these pathways. The qRT-PCRs were performed on intestines from flies crossed with enterocyte/enteroblast-specific driver 5966-GS, which was selected due to the extreme abrogation of longevity that was observed. For the initial qRT-PCR reactions, flies were placed on their respective diets (AL vs. DR) for 8 days, after which they were dissected. Dissection on day 8 after being sorted onto food, was chosen for these experiements because the rapid rate of death of the *PFD6*-crossed 5966-GS flies began around that day (see Figure 7B, C). All qRT-PCRs were standardized to housekeeping gene *RP49*, which is a standard housekeeping gene for *Drosophila* research (Lucchetta and Ohlstein 781-788). Because the objective of these assays was to determine the possible method of intestinal dysregulation, genes that were chosen included regulators and indicator genes of the apoptosis pathway, the cellular proliferation pathway, and the inflammation pathway. No significant changes were observed in the apoptosis or cell proliferation pathways, however, there was a change in the genes chosen for the inflammation pathway, these genes being Anti Microbila Peptide genes (AMPs) *Diptericin* and *Defensin*.

AMPs are expressed in reaction to infection and are a part of the innate immune response of most organisms (Dimarcq et al. 2507-2515). The two genes decided upon, *Diptericin* (*Dipt*)

and *Defensin* (*Def*), are robust AMP genes which are locally upregulated in response to infection (Dimarcq et al. 2507-2515). The qRT-PCR results indicated that the expression levels were increased by a 5 fold amount for *Defensin* (Figure 10A) and a 3 fold amount for *Diptericin* (Figure 10B), when *PFD6* was knocked down in ECs/EBs (compare solid columns to dotted columns). This data implies that loss of *PFD6* lead to increased permeability, which may have increased intestinal susceptibility to infection (as indicated by the upregulation of the AMP genes) which may have been a factor in decreasing lifespan.

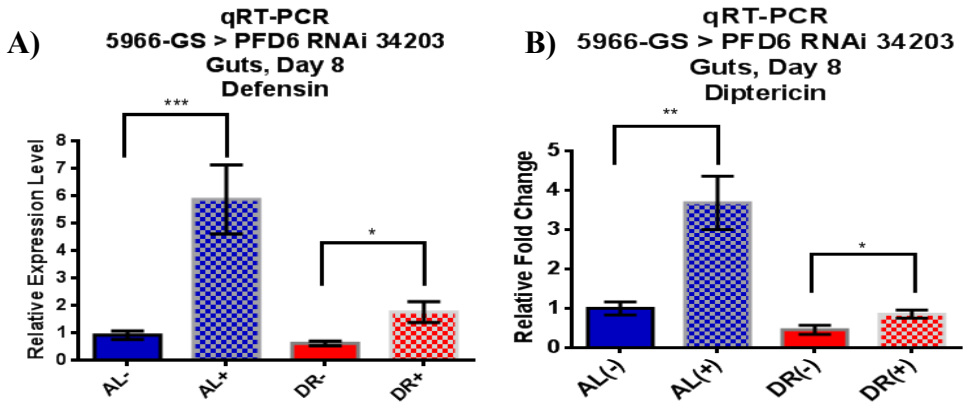

Figure 10: Quantitative Real Time PCR of dissected intestines of 8 day old flies.
Figure 10A: Relative expression of AMP gene *Defensin (def)* significantly increased in a diet-independent manner in knockdown of *PFD6* ($P < 0.0005$ on AL and $P < 0.05$ on DR). Figure 10B: Relative expression of AMP gene *Diptericin (dipt)* significantly increased in a diet-independent manner in knockdown of *PFD6* ($P < 0.0005$ on AL and $P < 0.05$ on DR). All qRT-PCRs are from intestinal tissue gathered on day 8 after sorting onto respective diets. All qRT-PCR graphs show Relative Fold Change, as relative to expression of housekeeping gene *RP49*.

Next, it was necessary to explore the biochemical pathways by which *PFD6* interruption lead to intestinal degradation. As stated before, there are a number of factors which mediate intestinal homeostasis, and may contribute to increased permeability, including increased apoptosis, dysregulation of repair, dysregulation of junction genes, and disruption of the

intestinal microbiota. In order to begin investigating by which mechanism loss of *PFD6* may increase intestinal permeability, I performed qRT-PCRs screening for apoptotic genes *Puckered* (Puc) (Figure 11A) and *Head Involution Defective* (*hid)* (Figure 11B), which are established markers for JNK signaling and apoptotic pathways, respectively (Shlevkov and Morata 451-460; Schetelig, Nirmala, and Handler 759-768). Expression of *puc* appeared to increase on the DR diet in flies, but not in the AL diet (compare dotted red to solid red in Figure 11A). Expression of *hid* appeared to undergo no significant change in expression.

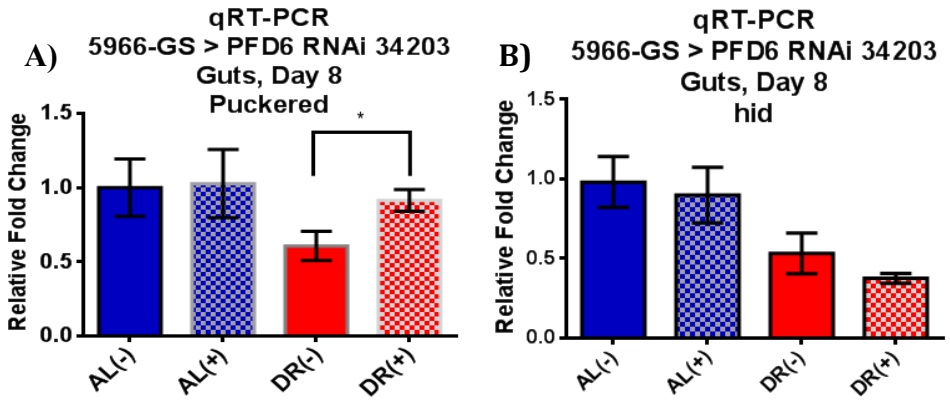

Figure 11: RT-PCR of intestines indicates that loss of *PFD6* in EC may contribute to increased apoptosis.
Figure 11A: Relative fold change in expression of apoptotic gene *Puckered (puc)* significantly increased in a diet-dependent manner in knockdown of *PFD6* ($P < 0.05$ on DR) in intestines from 8 day-old flies. Figure 16B: Relative fold change in expression of apoptotic gene *hid* appeared to have no significant increase in a diet-independent manner in knockdown of *PFD6* in EC/EB, in intestines from flies 8 days old.

My results showed that in addition to playing a role in regulation of apoptosis (Figure 11), *PFD6* also seemed to regulate compensatory proliferation by regulating the expression of *upd3*, a cytokine-expressing gene which regulates ubiquitous stem cell cycle and proliferation (Zoranovic, Grmai, and Bach) (Figure 12). Two RT-PCRs were performed on intestines dissected from *Drosphila* with loss of *PFD6* in EC/EB (5966-GS driver) at different days after

being on their diets, to view possible changes in expression of *upd3* over time. In the first RT-PCR, at day 8, no change in expression is observed. In the second RT-PCR on intestines from flies which had been on food for 14 days, there appears to be an increase in expression of *upd3* with loss of *PFD6* in the EC/EBs.

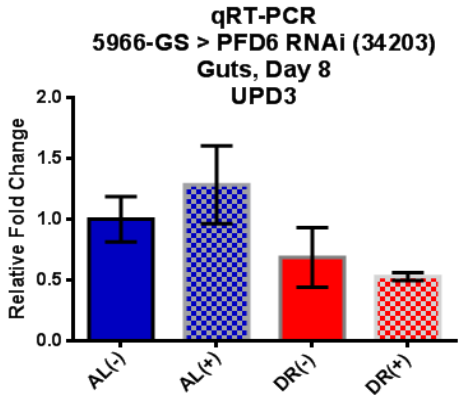

Figure 12: Relative fold change of cell cycle gene *upd3*.
Relative fold change of cell cycle gene *upd3,* observed in intestines taken from 8 day old flies, did not appear to be affected by knockdown of *PFD6* in Enterocyte/Enteroblast. Fold expression is standardized to expression of RP49.

Chapter 3: Identifying other subunits of the PFD complex and other genes that mediate gut permeability affected by Dietary Restriction.

Chapter 3.1: Smurf and pH3 assay screens reveal subunits *PFD6* and *URI* as potential co-modulators of intestinal permeability.

Having established cell specificity for *PFD6* effect, I thought it would be best to identify other subunits of the PFD complex, which could demonstrate similar regulatory capabilities on

intestinal homeostasis. The two methods used to screen co-modulator of *PFD6* were the Smurf assay, which allowed me to observe intestinal permeability, and the quantification of ISC proliferation via pH3 mitotic marker, which allowed me to observe the proliferation of intestinal stem cells in response to intestinal degradation. These two screens, done on various PFD subunits, enabled me to discover units with consistent correlations between knockdown of the subunit, changed intestinal permeability, and changed compensatory proliferation. Because the initial interest was only on the subunits, which showed a change in phenotype on low nutrient conditions (DR diet), the screens were performed only on said low nutrient conditions. Newly emerged adult flies were placed on 2 diets; a low nutrient diet without UAS-Gal4 inducing drug RU486 (blue columns), and a low nutrient diet with UAS-Gal4 inducing drug RU486 (red columns). Because flies placed on DR diets usually live longer than their high-nutrient counterparts, flies were kept on the food for 21 days, to attempt to observe a time point at which most genes would be expressing highest efficacy. In addition to knockdown lines, if Over-Expressor lines (OE) were available for the genes, they were also tested to observe possible effects on intestinal permeability/proliferation. Multiple variants of some genes were screened as commercially available, in order to observe which variants displayed the highest efficacy in knockdown or overexpression.

The Smurf screen for subunits that changed intestinal permeability, indicated a significant increase in intestinal permeability with only 2 subunits: *PFD6* knockdown (2 variants, showing at least 10 fold and nearly 100 fold increase), and *PFD6* over-expression (OE, or up-regulation) with a 5 fold decrease in permeability, and knockdown of *URI* (Figure 13A) with an approximate 2-fold increase in permeability. The subunits with consistent significant differences in proliferation included down-regulation of *I(2)35Cc* which showed an approximate 3 fold

decrease in proliferation; down-regulation of *Rept*- resulting in a doubling in proliferation, down-regulation of *PFD6* resulting in an approximate doubling in proliferation, up-regulation of *PFD6* resulting in a similar doubling in proliferation, down-regulation of *Pih1D1* which resulted in an approximate doubling in proliferation, down-regulation of *pont* which showed an approximate 2-fold decrease in proliferation, and down-regulation of *URI* which demonstrated an approximate 2-fold increase in proliferation (Figure 13B). The results of the two screens are summarized in Table 3.

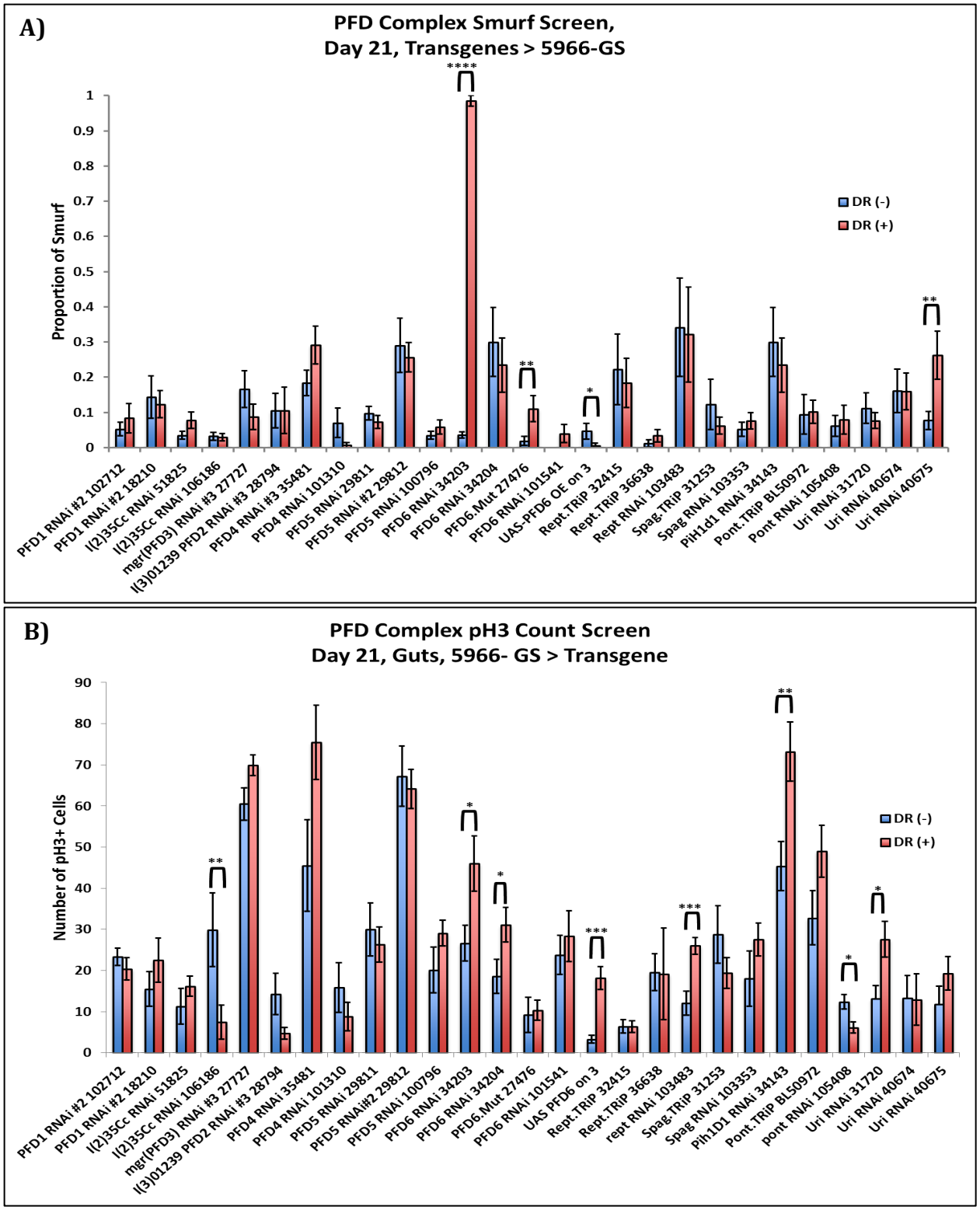

Figure 13: Co- Modulators of *PFD6* in Regulating Gut Permeability and proliferation

Figure 13A: Smurf assay of transgene and 5966 driver crossed flies, counted 21 days after occlusion. Genes which display a significant difference between wild type and gene knockdown include *PFD6* loss, Over Expression of *PFD6*, and *URI*.

Figure 13B: Phospho-Histone 3 positive cell counts of *Drosophila* intestinal tracts taken at day 21. Genes which display a significant difference between wild type and gene knockdown include *I(2)35Cc*, *Rept*, *PFD6* loss, Over Expression of *PFD6*, *Pih1D1*, *pont*, and *URI*.

Gene Variant Expressing Change	Change in Permeability (SMURF)	Change in Proliferation (PH3 Stain)	Change in Both
PFD6 RNAi	Increased Permeability	Increased Proliferation	Increased in Both
I(PFD2)35Cc RNAi	No	Decreased Proliferation	No
UAS-PFD6 OE	Decreased Permeability	Increased Proliferation	No
Rept RNAi	No	Increased Proliferation	No
Pih1D1 RNAi	No	Increased Proliferation	No
pont RNAi	No	Decreased Proliferation	No
URI RNAi	Increased Permeability	Increased Proliferation	Increased in Both

Table 5: Co- Modulators of PFD6 in Regulating Gut Permeability differentiated by a change in permeability detected with SMURF assay, change in proliferation detected by PH3 stain, or a change in both.

Chapter 3.2: Exploring the role of *URI* in Modulating Longevity and Intestinal Homeostasis on Dietary Restriction and Ad Libitum diets.

From the earlier screen performed, it was observed that there were only 2 subunits which, when lost, created a significant change in the intestinal permeability and the proliferation of the ISCs; *PFD6* and *URI*. It is conjectured that these subunits, if found to possess similar regulatory functions in the intestine, may co-modulate for additive, adverse, or synergistic effects as protein complexes. Therefore, it was necessary to first establish the possible parallels in function between *PFD6* and *URI*. As mentioned previously, *URI* has several unique regulatory functions, particularly in metabolism, in both the nucleus and the cytoplasm. Whole-body knockdown assays revealed that *URI* was indeed necessary for a normal lifespan, as when the gene was knocked down there was a significant abrogation of lifespan ($P < 0.0001$) (Figure 14A). However, this abrogation of lifespan appeared to be diet–independent in the whole body of the fly. When I limited the knockdown of *URI* in Enterocyte/Enteroblast, I observed a similar and even stronger diet-independent shortening of longevity (Figure 14B). Furthermore, repeated longevity assays revealed that the abrogation of lifespan was only significant in the AL diet (blue columns) (Figure 14C). Finally, an even more interesting result was observed when knockdown of *URI* occurred in ISC/EB in that this caused a slight extension of lifespan (Figure 14D), like the extension seen when *PFD6* was knocked down in ISC/EB (Figure 7C).

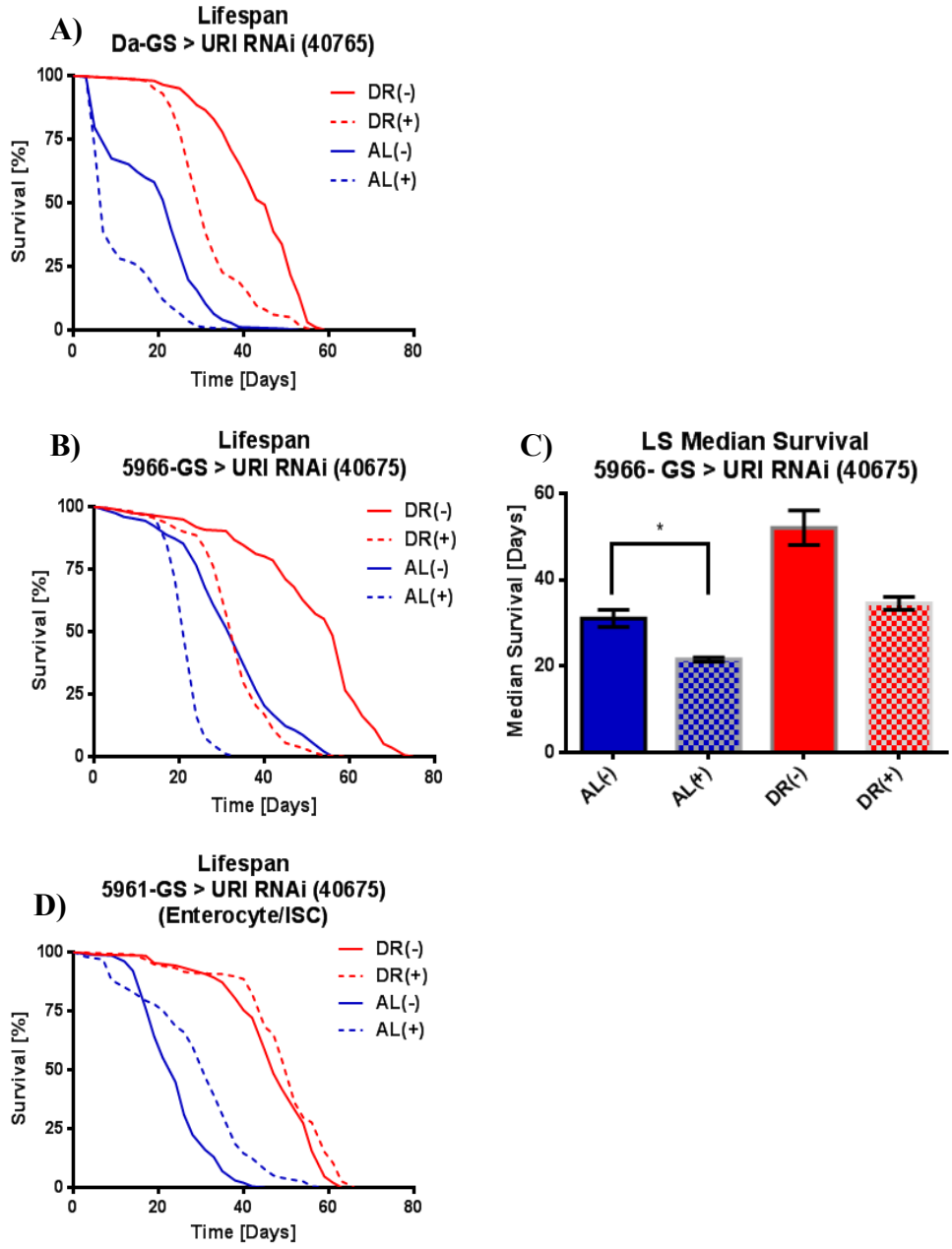

Figure 14: *URI* protein appears to be necessary in EC for lifespan.
Figure 14A: Lifespan assay of ubiquitous knockdown of *URI* subunit using Da-GS driver demonstrated an apparent diet-independent decrease in longevity. Figure 14B: EC/EB-specific knockdown of *URI* using 5966-GS driver demonstrated a significant abrogation of lifespan in a diet-dependent manner upon AL ($P < 0.05$) in lifespan assay. Figure 14C: Lifespan Median survival graph showing a significant difference in Median Lifespan of *URI* knockdown in EC/EB upon AL diet (compare solid blue to spotted blue columns) ($P < 0.05$). Figure 14D: EC/ISC-specific knockdown of *URI* seemed to extend lifespan in a diet-dependent manner, instead of abrogating in lifespan assay.

As with the *PFD6* subunit, after confirming that *URI* was necessary for longevity, it was necessary to verify that this was due to the dysregulation of intestinal homeostasis. Smurf assays were performed utilizing the same three drivers to separately down-regulate *URI* ubiquitously, in Enterocyte/Enteroblast, and in EB/ISC. The Smurf assay showed no significant change in permeability upon ubiquitous knockdown of *URI* (Figure 15A). EC/EB-specific knockdown of *URI* demonstrated significantly increased gut permeability in a diet-independent manner (Figure 15B, $P < 0.5$ in AL and $P < 0.005$ in DR). Finally, Smurf assay demonstrated no significant decrease, nor increase, in gut permeability upon down-regulation of *URI* in ISC/EB (Figure 15C).

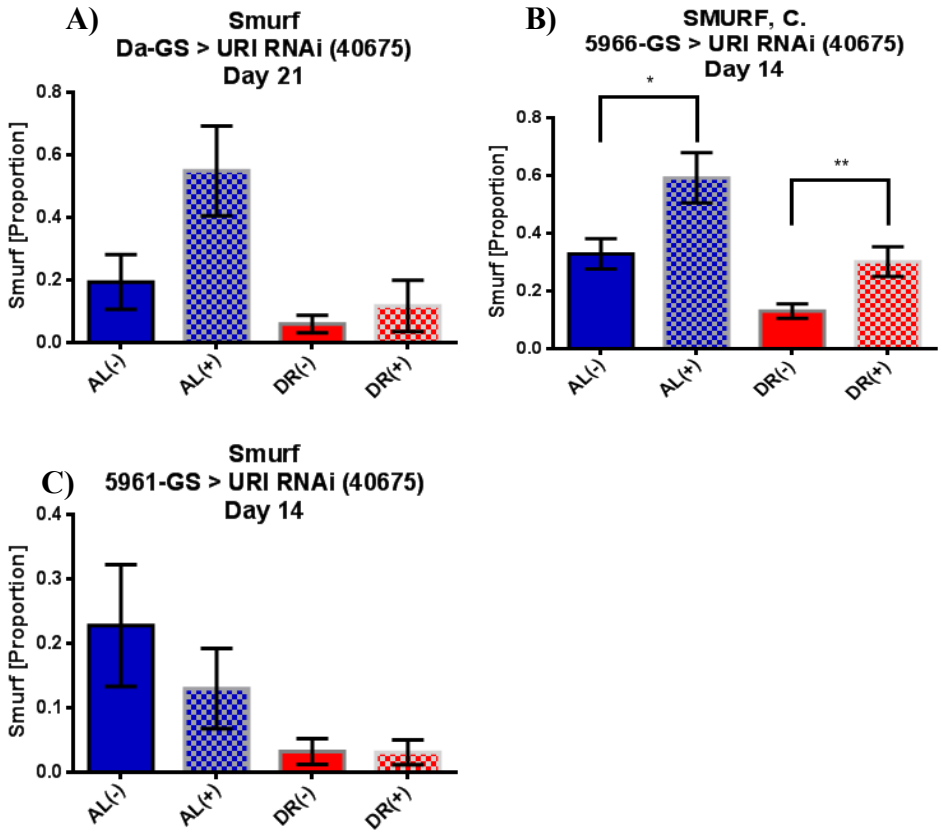

Figure 15: Intestinal permeability assay of down-regulation of *URI* in different cell types.
Figure 15A (Top): Smurf assay of whole whole-body knockdown of *URI* utilizing Da-GS driver shows that gut permeability does not significantly change in *URI* knockdown. Figure 15B (Center): Smurf assay of enterocyte/enteroblast specific knockdown of *URI* shows that gut permeability is increased in *URI* knockdown in a diet-independent manner ($P < 0.05$ and $P < 0.005$ for AL and DR diets, respectively). Figure 15C (Center): Smurf assay of ISC/EB specific knockdown of *URI* using 5961-GS driver shows no significant change in intestinal permeability.

As with the investigation of loss of *PFD6*, it is conjectured that a rise in intestinal permeability may cause, or be caused by a change in the stem cell proliferation due to loss of the protein. Thus, Phosphohistone3 Positive cell count assays for detection of replicating stem cells were performed on flies with loss of *URI* in the same three tissues/cells (whole body, EC/EB, and ISC/EB). In an attempt to match the timing of the change in lifespan phenotype displayed in Figure 14A, the pH3 assay for whole body loss of *URI* was performed 21 days after placing flies onto the diet, rather than 14. The Ph3 cell count assay showed a significant diet-dependent decrease ($P < 0.005$, and $P < 0.0005$) in stem cell proliferation with *URI* knockdown in the whole

body with Da-GS (Figure 16A). However, because proliferation may occur at different times in the many tissues in the fly, it was necessary to see if proliferation was increased in the intestine acting as a repair mechanism to inhibit the increased permeability observed before (Figure 15B). To answer if this might be occurring, additional pH3 stains were done in flies with EC/EB-specific and EB/ISC-specific downregulation of *URI*. In the EC/EB-specific *URI* knockdown, I observed higher numbers of pH3-positive cells on the DR, which indicated a significantly higher stem cell proliferation with loss of *URI* in the DR diet (Figure 17B, red columns, $P < 0.05$). Finally when down-regulating *URI* in ISC/EB, a significant decrease in the number of proliferating stem cells in the AL diets, as seen by decreased numbers of pH3-positive cells (Figure 17C, blue columns, $P < 0.05$) was observed. There was an interesting correlation between the increase in the proportion of Smurf (intestinal permeability) seen in the ISC/EB-specific downregulation of *URI* (Figure 16C), and the increase in the proliferation of Stem cells with loss of *URI* in said same tissue, which lead to the conclusion that proliferation in the context of knockdown of *URI* in the EC/EB was occurring as a response to damage to the intestine.

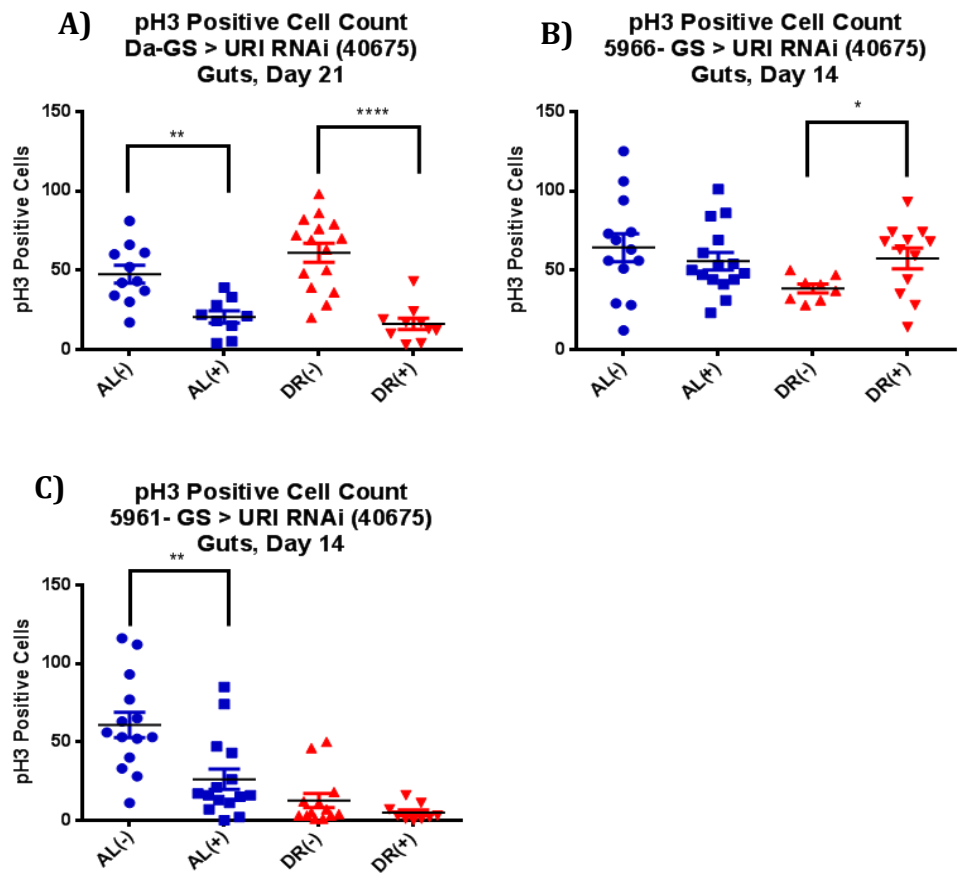

Figure 16: Loss of *URI* in each tissue appears to change proliferation differently in tissue type.
Figure 16A: Proliferation assay via Phosphohistone3 Positive Cell count of 21 day-old flies with ubiquitous down regulation of *URI* subunit using Da-GS. There was a significant decrease in the number of pH3-positive cells in *URI* knockdown on both diets, AL reduction ($P < 0.005$) and DR reduction ($P < 0.00005$). Figure 16B: Enterocyte/Enteroblast-specific knockdown of *URI* using 5966-GS demonstrated a significant increase in proliferation upon the DR diet ($P < 0.05$). Figure 16C: Intestinal Stem Cell-specific knockdown of *URI* in ISC using 5961-GS decreased proliferation on AL (blue circles and blue squares) diet in a significant manner ($P < 0.05$).

Discussion:

Chapters 1 and 2: The Role of *PFD6* knockdown on Longevity, Intestinal Permeability, and Proliferation.

In Figures 7A and 7C, one sees that there is a diet-independent shortening of lifespan, as supported by the median survival graph Figure 7D. This serves to confirm that *PFD6* is essential for longevity in EC/EB, and that it mediates longevity through the disruption of the intestinal EC/EB cells, and the increase in intestinal permeability, as shown in Figure 8A and 8B. However, as stated before, there are 3 possible pathways by which loss of *PFD6* may cause disruption of the epithelium; unbalanced increase in apoptosis, dysregulation of the proliferation (repair) response to apoptosis/necrosis (non-programmed cell death), or dysregulation of the junction genes which mediate cell-cell interactions in the tissue of the epithelium. We attempted to map the pathways responsible for this dysregulation of intestinal homeostasis by performing qRT-PCR on disrupted guts (particularly in the EC/EB-specific knockdown of *PFD6*). My results showed that there appeared to be a increase in regulation of apoptotic pathway gene *puc* on the DR diet (Figure 11A, red columns). However, this result was not duplicated in regulation of the other apoptosis-related gene, *hid*, which showed no significant change in expression level (Figure 11B). This could be due to 2 possibilities; 1) loss of *PFD6* in EC/EB does not activate pro-apoptotic pathways and cause damage to the intestine via cell apoptosis, 2) the time frame of our experiments was not at a date where loss of *PFD6* had yet to make a significant change in phenotype. Thus, one would turn to the second of the possible mechanisms; disruption of the proliferation pathway. However, no change was observed in ISC proliferation upon knockdown of *PFD6* (Figure 9B), which correlates with no change in expression of cell cycle marker *upd3* (Figure 12A). Once again, there are two possible interpretations of this data; 1) no disruption to

the proliferation pathway is being observed, 2) the proliferation signaling pathway is being disrupted, however, this disruption is occurring at pathway more downstream of *upd3* expression (such as *p53)*, and therefore could not be observed with the RT-PCRs performed here. This second reason could explain why the EC/EB knockdown of *PFD6* results in such a comparatively high proportion of Smurf, yet remains undetectable. However, future steps of this research would do well to check biochemical targets downstream of each of the above listed genes (*puc, hid, upd3*), to verify these results. If no correlative results are obtained, it could very well be that loss of *PFD6* disrupts the membrane by disruption of the Junction gene pathways, such as by dysregulating genes such as *Junctional Adhesion Module (JAM)*, or by disruption of the microbiota. However, this is all particular to the loss of *PFD6* in EC/EB. This does not explain the phenotypes observed with loss of *PFD6* in ISCs.

Now, what is interesting is that ISC/EB-specific down-regulation of *PFD6* appears to not share the abrogation of lifespan (Figure 7D and 7E). This could possibly be due to the fact that ISCs make up less than 10% of the intestinal tissue; therefore, the collapse of that cell type is not significantly detrimental to the intestinal epithelium and the fly as a whole. This argument is supported by the Smurf data, which shows no significant change in the proportion of Smurfed cells upon change in diet or knockdown of the subunit, and has comparatively low levels of Smurf when compared with the other cell types (Figure 8C). With the 5961-GS knockdown of *PFD6* in ISCs, I was expecting to see severely decreased levels of ISCs. However, the proliferation assay for the ISC/EB-specific knockdown of *PFD6* only shows a significant decrease in the number of proliferating stem cells on the AL diet (Figure 9C). This could be interpreted in 2 ways: 1) that *PFD6* down-regulation disrupts the regular formation of ISCs in the intestine, which makes sense given the increase in permeability in EC/EB-specific

knockdown seen in Figure 8B and the apparent lack of compensatory proliferation for the damage in Figure 9B; or 2) proliferation is lower with knockdown of *PFD6* in ISC/EB because diet-induced damage, or cellular disruption due to loss of *PFD6*, is similarly reduced, which appears to correlate somewhat with the lack of high amounts of Smurf flies in ISC/EB down-regulation of *PFD6* (Figure 8C) and also with the apparent, although slight, extension in lifespan on the AL diet (Figure 7C). However, a lack of information on the signaling pathway of ISC/EB-specific down-regulation of *PFD6* prevents the current possibility of understanding this.

The qRT-PCR data for EC/EB-specific down-regulation of *PFD6* tells a more simplistic story. Knockdown of *PFD6* possibly results in higher apoptosis (Figures 11B and 11C), which could be the cause for the increase in intestinal permeability (Figure 8B). This could be associated with higher chances of infection, as larger 'gaps' appear in the intestinal epithelium which can make it easier for larger cellular bodies and pathogens to pass through (Figure 10), which could explain the abrogation of lifespan (Figure 7B). All of this data is also depicted in the model presented in Figure 14.

However, there were a few challenges presented by some of the data and experiments to be evaluated or overcome. One of the first issues that this research faced was the inconsistency of the intestinal permeability (Smurf) and proliferation (pH3) assays**.** Often these assays would have to be repeated 2 to 3 times, and even then results were not always consistent. One of the potential causes for this, in regards to the Smurf assay, is due to the early death of *Drosophila* due to unforeseen factors, such as drowning in their food, which sometimes can obfuscate the significance of the data, or possible fungal infection of the food, which can result in increased intestinal infection or permeability in the flies. With regards to the pH3 stains, this requires careful handling due to the fragility of the intestines, the damaging of which can drastically

change the amount of countable cells. This difficulty I managed to rectify by simply repeating the experiment, providing an ample amount of data for significance.

Additionally, the lifespan assays, particularly when using the Da-GS driver, are also a concern because the Da-GS driver is a very strong driver. It is often cited in my lab for causing whole body damage, which can be difficult to distinguish from the damage due to gene knockdown. To avoid this, several control experiments were done crossing Da-GS with W1118 flies (a control strain), to expose potential significant differences in the lifespan phenotypes that were used. Because the Da-GS driver causes such strong whole body expression, by crossing with a control strain (W1118), I can observe if there is a significant difference in the lifespans of flies expressing or not expressing the Da-GS driver. If there had been a lesser or stronger shortening of lifespan when the cross was done with W1118, it would have shown that the Da-GS driver itself was affecting the research. However Figure 7B featuring a control assay using W1118 demonstrated no significant change from the shortening of the transgene flies, indicating that the Da-GS driver is not acting as an additional cause of damage in these experiments.

Also of concern was the time frame for experiments. As the reader may note, many, but not all of the assays, were performed on days 14 or 21. Consistency in the timing of experiments is always a concern with research on aging, but particularly in flies where every day can amount to a significant portion of a flies' lifespan. Days 14 and 21 were selected as being the best "average" time points at which to collect and observe median lifespans, intestinal permeability, and proliferation assays, without suffering from significant loss of numbers of flies due to transgene knockout or other lethal phenotypes. Thus, it was not statistically sensible to compare the days at which time points were collected for two different genes, as the phenotypic effects of the genes may peak at different points in the *Drosophila* lifespan. However, for the sake of side-

by-side comparison, day 14 was chosen as a 'standard' time to compare data due to the evidence that this is the time frame when *PFD6* appears to significantly alter the lifespan when being ubiquitously down-regulated using the Da-GS driver (Figure 7A). Day 8 was chosen for the qRT-PCRs because they were EC/EB-specific down-regulated, which in the lifespan assay (Figure 7B) showed a most dramatic decrease in lifespan between days 6-10. In this way, we also had an additional time point to view possible early expression of these genes than just at day 14, and any effect on them that may occur due to *PFD6* down regulation. Future experimentation would be best served by having assays done at time points appropriate for each phenotype, as was done with some of the PCRs. However, this would require Smurf and pH3 assays to be performed at multiple time points for knockdown or over expression of each subunit, and was not feasible for the time frame of this research.

Other good future directions of research would include examining the possible effects of down-regulation of *PFD6* on the expression of tight-junction genes, which also play a crucial role in intestinal maintenance (Arrieta, Bistritz, and Meddings 1512-1520; Lee 11-18). One possible way of confirming this possibility would be by performing further qRT-PCRs for junction genes at different time points, or taking a kinetics approach to model potential interacting domains between junction proteins and the domains of *PFD6* . This could also be done using epistasis experiments. For this to occur, it would be necessary to have 3 simultaneous intestinal permeability assays; 1 to demonstrate that loss of a particular junction gene increases intestinal permeability (established in literature), a second permeability assay with over-expression of *PFD6* to act as a standard against which to compare the third permeability assay, in which a tight junction gene could be down-regulated and crossed with a line over-expressing *PFD6*, in an attempt to rescue the afore mentioned increased permeability. Epistasis

could also be done with the subunit *URI*, from the second chapter of my research, because the possibility of *URI* to act as a co-modulator for *PFD6* and vice-versa, has now been somewhat established.

These results led to the conclusion that *PFD6* did have significant effects on longevity as mediated by intestinal permeability. Figure 17 is a conjectured pathway by which *PFD6* is able to mediate intestinal homeostasis and how this in turn affects longevity. On the left, when PFD6 is expressed at Wild-Type (standard) levels, dietary stress may create damage to the ECs. However, the standard intestinal repair mechanism is functioning. Cytokines are released from damaged ECs, inducing ISC proliferation response into EBs, which differentiate into ECs to replace the damaged/disrupted EC cells, and maintains an intact intestine with low intestinal permeability to retain standard longevity. However, when *PFD6* is lost in the ECs (shown on the right side image), dietary-stress induced damage does not result in the release of cytokines. This lack of cytokine activity keeps ISC proliferation at standard levels when more proliferation is necessary to compensate for damaged cells in the tissue. Therefore, over time, the tissue degrades or is otherwise disrupted, resulting in increased permeability of the intestine, and lowering longevity. Should this model prove translatable to mammalian models, there are implications that *PFD6* may be a candidate target gene/protein for proteopathic disorders, particularly in the intestine.

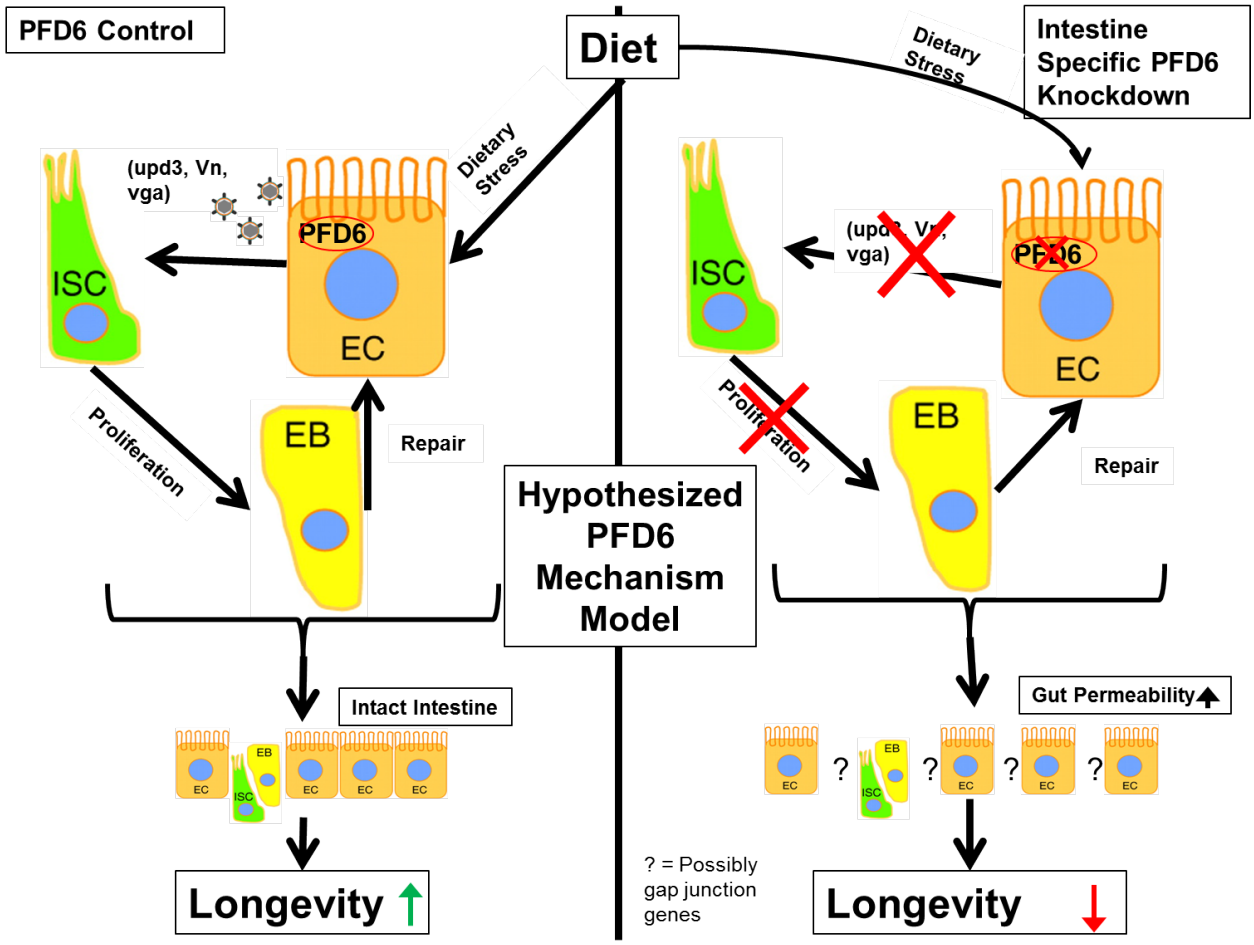

Figure 17: Conjectured pathway of the means by which *PFD6* affects intestinal homeostasis and in turn, longevity.
Left: *PFD6* Control shows the regular pathway. Damaged Enterocytes release secreted factors inducing proliferation of ISCs, which proliferate into EB's, which differentiate into EC's to maintain an intact intestinal epithelium;
Right: Loss of *PFD6* interrupts the EC→ ISC → EB pathway, leading to an increase in Intestinal Permeability, and decrease in longevity.

Chapter 3: *URI* is another subunit of the PFD complex that mediates gut permeability.

Chapter 3.1: *URI* is the only other notable co-modulator of *PFD6*, as identified by Smurf and pH3 assays.

Having established the necessity for *PFD6* in longevity and intestinal health, it seemed prudent to search for possible co-modulators of *PFD6* from within the PFD complex. As stated before, Smurf assay and proliferating cell count assay (with pH3 staining) are the methods chosen, mostly because longevity assays for the knockdown of each subunit could not feasibly be completed within the time frame for this thesis. Additionally, it was decided that because co-modulators for DR conditions were what were desired because we were looking for subunits that would activate pathways expressed mostly on low nutrient diets, results for AL experiments would also be excluded. Because DR usually extends the median lifespan, it was decided that the day to gather results for comparison would not be day 14, as with most assays, but Day 21 to hopefully catch the changes in phenotype that might be similarly extended, for example, a drastic change in phenotype was seen between days 20-30 in flies on DR food in Figure 7A, and between days 30-40 in flies on DR in Figure 7C. One of the other difficulties about the use of these assays is that some of the flies were difficult to culture. The usual sample size for the assay is around 200 flies; however, some of these fly strains displayed low fecundity, or low survivability, so for some of these assays only around 150 flies could be procured. Thus, it would be good to repeat these results to increase the significance of the data and decrease the margins of error.

However, some of the limitations of using one assay versus the other was mitigated by using both screens to view the effects of down regulation of the individual subunits, and further studying only the subunits which showed a significant change in both of them. With this

approach, we identified subunits *PFD6* (acting as our comparable control) and *URI* (Figure 14 and Table 3). The fact that there was a change in both permeability and proliferation on DR diet informs me of two important facts: 1) *PFD6* and *URI* both are both essential for proper functioning, else they would not show a difference on both of these screens; and 2) *URI* is also capable of regulating intestinal functions independent of the overall *PFD* complex similar to *PFD6*. For both of these reasons, I decided that *URI* would be the next subunit to warrant research and investigation.

Chapter 3.2: Exploring the role of *URI* in Modulating Longevity and Intestinal Homeostasis on Dietary Restriction and Ad Libitum diets.

With the discovery of the subunit *URI* as a possible co-factor for *PFD6*, my first order of business was to establish *URI*'s own role in intestinal health and longevity independent of the PFD complex. In order to allow me to compare the results from *URI* to those of *PFD6*, I elected to use the same assays, with many of the same time points as were done for *PFD6*, starting with the longevity assay.

However, in the course of my experiments, some of my results were compromised by fungal infection of the fly food, which limited my ability to perform only a single lifespan assay of loss of *URI* in the whole body (Da-GS) and in the ISC (5961-GS), hence why I was only able to gather sufficient lifespan data to make a median lifespan graph for the loss of *URI* in the EC (Figure 15C). This lessens the significance of my results of loss of *URI* in other tissues, however, from the data procured.

The longevity assay data for the *URI* knockdown demonstrated that it is indeed necessary for longevity, as fly lifespans were decreased when it was knocked down both ubiquitously

(Figure 15A) and in the AL diet when lost in EC/EB (Figure 15B and 15C). Similar to the *PFD6* knockdown in EC/EB, there is a significant increase in the intestinal permeability of flies when *URI* is down-regulated in EC/EB (Figure 16B), though there is no parallel significance in the knockdown of *URI* ubiquitously (Figure 16A). Recalling that there was a significantly higher proportion of Smurfed flies in the DR diet than the AL diet for loss of *URI* in EC/EB (see Figure 16B), it is conjectured that the similar increase in pH3-postitive cells with loss of *URI* in the EC/EB, indicated a response to damage, rather than a protective effect. The difference between the two is that a protective effect is generated before the onset of damage, and can prevent either some or all damage from occurring, a response to damage is initiated after the damage has occurred, and can rarely mitigate all of the negative effects of damage. Thus, it could be conjectured that though knockdown of *URI* in EC/EB leads to intestinal damage, there is a protective effect generated elsewhere in the intestine in response, mitigating the permeability and decrease in lifespan. However, this needed to be confirmed via observation of the effects on proliferation.

As with *PFD6*, I stained for proliferating stem cells to observe one avenue of damage compensation. In knocking down *URI* ubiquitously using Da-GS, I observed a significant decrease in the amount of proliferating cells on both DR and AL diets (Figure 17A). My first thought was that this indicates that the damage being done to the intestine is being somehow mitigated, seeing as how the lifespan does not have as severe an abrogation as when *URI* is knocked-down in EC/EB (compare Figure 15A and 15B). However, down-regulation of *URI* in EC/EB resulted in an decrease in proliferating stem cells on DR (Figure 17B), which could potentially indicate damage. In the context of the longevity assay, which showed a significant decrease only on AL diet with EC/EB knockdown of *URI* (Figure 15B and 15C), this could

indicate that *URI* knockdown dysregulated other proliferative pathways, such as EGFR and JNK (Ayyaz, Li, and Jasper 736-748; Biteau and Jasper 1045-1055), which may otherwise be properly functioning. This makes sense given my proliferation cell counts of down-regulation of *URI* in ISC/EB (Figure 17C), which shows a significant decrease in proliferation upon only the AL diet. In fact, the proliferation assay appeared to correlate well with the proportion of Smurf shown in Figure 16C. All of this also demonstrated lower levels of damage and less necessary compensation, which made sense given the longevity results for ISC/EB knockdown of *URI* (Figure 15D), showing that ISC/EB-specific down-regulation of *URI* appears to rescue (or at least does not decrease) the median lifespan of the flies, similar to *PFD6* down-regulation in ISC/EB (Figure 7D and 7E). However, these results still bear further investigation, particularly into the other biochemical mechanisms likely at work.

Finally, one of most important issues facing this research is its translatability to other eukaryotic organisms. Though PFD is a phylogenetically conserved complex between Archaea and Eukaryotes (Geissler, Siegers, and Schiebel 952-966), the subunits studied here are still orthologues of the human subunits, and do not reflect the true nature of the complex as found in human. However, by understanding this complex in flies, we hope to translate our understanding into other eukaryotic models.

Closing Paragraph

This study explored some the novel functions served by two of the subunits of the PFD complex, in *Drosophila* lifespan extension and intestinal homeostasis. To answer this question, the functions of *PFD6* and *URI* in maintaining intestinal homeostasis were categorized. After this, it was determined whether *PFD6* and *URI* regulate longevity through a somatic cell lineage, or a stem cell lineage in flies. It was confirmed that the more significant change in phenotype occurred in down-regulating *PFD6* and *URI* in Enteroctyes/Enteroblasts, and thus the research was pursued in that avenue to map the changes in intestinal homeostasis and lifespan regulated by these proteins. Finally, several likely downstream targets of the subunit *PFD6* were identified, allowing for investigation of the mechanisms utilized in knockdown of the *PFD6* subunit to have the effect on *Drosophila* homeostasis. These experiments allowed us to better qualify the importance of the Cochaperon protein Prefoldin, and may have expanded upon some of the current understanding of the functions that *PFD6* and *URI* play in proteostasis, particularly where it may affect intestinal function and organism lifespan. In addition, because the PFD complex is very important to protein function and maintenance, there is the possibility that these subunits could be valuable drug targets to regulate gut health and longevity.

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